Improvement of Lentil (*Lens culinaris* Medik.) through Genetic Transformation

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Dedicated to my beloved parents
And
My respected teachers

ABSTRACT

Work title: Improvement of Lentil (*Lens culinaris* Medik.) through genetic transformation.

Hashem, Rehana

The future agriculture will depend more on legume crops because they all have high energy and high protein production for human and animal nutrition as well as amino acid profiles complementary to those of other crops, mainly cereals. The unique symbiotic ability of legumes is to use atmospheric nitrogen for plant growth makes them preferable crops for sustainable agriculture. Lentil is the 2nd most important grain legume that gained worldwide economic importance as a source of protein (25.5 – 28.31 %). In addition, it is also suitable as a rotation crop to replenish soil nitrogen levels. It is a crop of cooler temperature and is widely grown in the temperate zones of the world. The production of lentil is usually considerably below the established yield potential as this crop is very sensitive to particular biotic and abiotic stresses. The most serious biotic attribute constrain in lentils are the foliar diseases such as *Ascochyta* blight, rust, *Stemphylium* blight and *Botrytis* grey mold. Yield stability and productivity and the value of lentil could be greatly increased by the introduction of stably inherited traits such as pest and disease resistance, herbicide resistance or improved protein quality. These traits are not available in natural populations of near relatives of cultivated lentils, but current advances in plant genetic engineering provide a potentially powerful tool for achieving these goals by an alternative mean.

The aim of the present study is therefore, the establishment of a reproducible and efficient transformation system for *Lens culinaris* Medik which is suitable for the insertion of agronomically desirable genes to overcome the limitations imposed by traditional breeding process. Along with this, another objective is to explore a simple marker free transformation system. Antibiotic resistance genes (e.g. *npt*II*, hpt*) or herbicide resistance genes (e.g. *bar*) are essential for selectively propagating transformed cells and tissues. However, subsequent maintenance of markers is unnecessary. Elimination of markers is advocated since it theoretically can not be excluded that antibiotic or herbicide resistance genes may be transferred to pathogenic bacteria or weeds, although the likelihoods are extremely low.

The *Bari Musur* variety, BM4 was selected through its regeneration performance. Decapitated embryos with single cotyledon discs were selected as explant for transformation. The *Agrobacterium* strain EHA- 105 with the plasmid pSCP1 was used for transformation. The plasmid was harbouring the selectable marker gene *bar*, which encodes the enzyme phosphinothricin acetyltransferase (PAT) and a *pgip* gene from raspberry (*Rubus idaeus* L.),

coding for polygalacturonase inhibitory protein. The expression of this recombinant gene can confer resistance against fungal pathogens (*Colletotrichum, Botrytis etc)*. The total procedure from seed to seed was between 2.5 - 4 months until getting transgenic lentil seeds. Transformation efficiency was found to be about 29%. For assessing the possibilities to develop a marker free transformation system, the *bar*-gene was removed and *PGIP* gene was kept in the T-DNA cassette before carrying out transformation work. Transformation with the new construct gave us a transformation success rate of 35% as estimated from the T_0 clones. The first analysis of a transformation rate of 35% will be confirmed by further analysis of the progenies. On the functional level, the plants were analyzed via a semi-quantitative polygalacturonase-inhibition assay. Activity of the *pgip* gene against *Colletotrichum lupini*, *C. acutatum*, *Botrytis cinerea* was tested. It was shown**,** the established method could provide a powerful tool to achieve markerfree transgenic lentil plants.

Keywords: *Agrobacterium*, transgenic lentil, polygalacturonase inhibiting protein, antifungal resistance, marker-free transformation.

Zusamenfassung

Arbeitstitel: Verbesserung der Linse (*Lens culinaris* Medik.) durch genetische Transformation
Hashem, Rehana

Die Bedeutung von Leguminosen in der Landwirtschaft wird in Zukunft aufgrund ihres hohen Energie- und Proteingehalts für den Bereich der menschlichen und tierischen Ernährung steigen. Zusätzlich enthalten Hülsenfrüchte, im Vergleich zu anderen Feldfrüchten wie z.B. Getreide, ergänzende essentielle Aminosäuren. Die einzigartige Fähigkeit von Leguminosen über die Symbiose mit Rhizobien atmosphärischen Stickstoff für ihr Wachstum zu nutzen, macht sie zu bevorzugten Saaten in der nachhaltigen Landwirtschaft. Durch den hohen Proteingehalt (25,5-28,31%) gehört die Linse zu den wichtigsten Hülsenfrüchten weltweit. Des weiteren spielt sie eine wichtige Rolle in der Fruchtfolge zur Regeneration des Stickstoffs im Erdboden. Angebaut wird die Linse hauptsächlich in gemäßigten Zonen. Aufgrund der hohen Sensitivität gegenüber biotischen und abiotischen Stressfaktoren liegt die Produktivität der Linse oft weit unterhalb des möglichen Ertragspotentials. Eines der größten Probleme beim Linsenanbau stellen Blattfleckenkrankheiten wie *Aschochyta* und *Mycospherella* dar, aber auch Rost (*Uromyces*) und Grauschimmel (*Botrytis*), und bodenbürtige Erreger wie *Aphanomyces euteiches* sind von Bedeutung. Durch die Integration stabil vererbter Merkmale wie Schädlings- und Krankheitsresistenzen, Herbizidresistenz oder erhöhte Proteinqualität könnte die Ertragsstabilität und damit die Produktivität der Linse deutlich verbessert werden. Diese Eigenschaften sind im Genpool der Linse nicht vorhanden. Die Pflanzenbiotechnologie bietet hier leistungsfähige Werkzeuge für das Erreichen dieser Zielsetzungen.
Das Ziel der vorliegenden Arbeit ist die Etablierung eines reproduzierbaren und effizienten Transformationssystems für *Lens culinaris* Medik., welches die Integration von `Genes of interest´ (GOI) ermöglicht und somit die Grenzen der traditionellen Züchtung überwindet.
Des weiteren soll ein markerfreies Transformationssystem entwickelt werden. Antibiotika- oder Herbizidresistenzgene sind für die Selektion transformierter Zellen und Gewebe
Sehr nützlich, zur weiteren Kultivierung jedoch nicht unbedingt erforderlich. Die Elimierung der Markergene ist erstrebenswert, da es theoretisch nicht ausgeschlossen werden kann, dass Antibiotika- und Herbizidresistenzen auf pathogene Bakterien oder Wildkräuter übertragen werden könnten, obwohl die Wahrscheinlichkeit hierfür sehr gering ist.
Aufgrund der Regenerationsfähigkeit wurde die Sorte Bari Musur (BM4) ausgewählt. Für die Transformation wurden dekapitierte Embryonen mit einzelnen Kotyledonen als Explantate eingesetzt.

Für die Transformation wurde der Agrobakterium Stamm EHA 105, mit dem Plasmid pSCP1D verwendet. Das Plasmid enthält sowohl das selektierbare Markergen *bar*, welches für die Phosphinothricin Acetyltransferase (PAT) kodiert, sowie ein *pgip* Gen der Himbeere (*Rubus idaeus* L.), das das Polygalakturonase inhibierende Protein kodiert. Die Expression dieses rekombinanten Gens kann eine erhöhte Pilzresistenz gegenüber Pathogenen wie z.B. *Colletotrichum* und *Botrytis* bewirken.

Die gesamte Kulturdauer von Aussaat der Samen und Transformation bis zur Gewinnung von transgenen Linsensamen betrug 2,5 bis 4 Monate. Dabei konnte eine Transformationseffizienz von etwa 29% festgestellt werden.

Zur Entwicklung eines markerfreien Transformationssystems das *bar* Gen aus dem Plasmid eliminiert, so dass die T-DNA nur noch das *pgip* Gen enthielt.

Aufgrund der ersten molekularen Analysen ergab sich eine Transformationsrate von 35%, diese Ergebnisse sollen in zukünftigen Analysen der Nachkommenschaften noch verifiziert werden.

Auf funktionaler Ebene erfolgte die Analyse der Pflanzen durch einen semi-quantitativen Agarose Diffusionstest. Getestet wurde die Aktivität des *pgip* Gens gegenüber pilzlichen Polygalacturonasen von *Colletotrichum lupini*, *C. acutatum* und *Botrytis cinerea*.

Das neu etablierte Transformationssystem könnte also eine effektive Möglichkeit zur Herstellung gentechnisch verbesserter markerfreier Linsen darstellen.

Keywords: *Agrobakterium*, transgene, Linsen, PGIP, Pilzresistanz, markerfrei transformation.

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ABBREVIATIONS

appx.	Approximate
B5	B5 basal medium
BAP	6-**b**enzyl-**a**mino-**p**urine
bar	Bialaphos resistance gene
bp	**B**ase **p**air
CaMV	**Ca**uliflower **M**osaic **V**irus
cDNA	**C**omplementary **DNA**
CTAB	**C**etyl**t**rimethyl**a**mmonium**b**romide
dist.	Distilled
DEA	**Die**thanol-**A**mine
dNTP	**D**eoxy **n**ucleoside **t**ri-**p**hosphate
EDTA	**E**thylene**d**iamine**t**etraacetic **A**cid
EtOH	Ethanol
FW	**F**resh **w**eight
g/l	**G**ram per **l**itre
GOI	**G**ene **o**f **i**nterest
GUS/gusA	β-glucuronidase(enzyme/gene)
IBA	**I**ndole **b**utyric **a**cid
h	**H**ours
incl.	**Incl**usive
L	**L**iter
LB	**L**eft **b**order-Sequence
LRR	**L**eucine-**R**ich-**R**epeats
LS	**L**ongitudinal **S**ection
M	**M**ole
max.	**Max**imum
mg	**M**illi**g**ram
mg/l	**M**illigram per **l**itre
min	Minute
mM	**M**illi**m**ole
MS	**M**urashige & **S**koog
µl	Microlitre
µM	Micromole

ng	**n**ano**g**ram
NAA	α-**N**aphtalene **a**cetic **a**cid
*npt*II	**N**eomycin-**p**hospho-transferase(gene)
OD	**O**ptic **D**ensity
PAT	**P**hosphinothricin-**A**cetyl**t**ransferase
PCR	**P**olymerase **C**hain **R**eaction
PG	**P**oly**g**alacturonase
PGIP	**P**oly**g**alacturonase-**i**nhibitory **P**rotein
ppt	**P**hos**p**hino**t**hricin
PTGS	**P**ost **t**ranscriptional **G**ene **S**ilencing
RB	**R**ight **b**order-Sequence
Ri-pgip	**P**oly**g**alacturonase-**i**nhibitory **P**rotein of strawberry (*Rubus idaeus*)
rpm	**R**otations **p**er **m**inute
T_0, T_1, T_2, T_3	**T**ransgenic lines, (first, second and third generation inbreed progeny)
T-DNA	**T**ransferred **DNA**
TGS	**T**ranscriptional **G**ene **S**ilencing
Ti-Plasmid	**T**umour-**i**nducing plasmid
v/v	**V**olume per **v**olume
vir-Region	**Vir**ulence- Region
w/v	**w**eight per **v**olume

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1. INTRODUCTION

Today's agriculture continues to depend on legume crops because they all have high energy and high protein production for human and animal nutrition as well as amino acid profiles complementary to those of other crops mainly cereals. The unique symbiotic ability of legumes is to use atmospheric nitrogen for plant growth makes them preferable crops for sustainable agriculture. In addition, legumes are also diverse in both their adaptations to most of the world's agricultural and natural habitats (Oram and Agocaoili, 1994, ICARDA 1998, 2000, Wheeler, 2000).

Grain legumes are commonly known as pulses and are cultivated throughout the world. The pulses are amongst the earliest food crops to be cultivated by man. They have been treated as one of the most important source of dietary protein, especially in Asia, Latin America and Africa. Pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.) and chickpea (*Cicer arietinum* L.) are the main grain legumes grown mainly as dry seed for human consumption and animal feed or as vegetables. Lentil is preferred over the other pulses by consumer's preference all over the world. This important grain legume gained worldwide economic importance as a source of protein for human and animal nutrition. The importance of lentil lies in the fact that it is a major source of good quality protein in the common diet as the protein content can reach 24 -30%. The production of lentil is usually considerably below the established yield potential as this crop is very sensitive to particular biotic and abiotic stresses (Erskine *et al.*, 1994). The most serious biotic attribute constrain in lentil are the foliar diseases such as *Ascochyta* blight, rust, *Stemphylium* blight and *Botrytis* gray mold. Root rot caused by *Sclerotinia* and wilt by *Fusarium* sp. are the other two diseases responsible for yield loss. Rainfall and drought are to be listed as important abiotic constrains for lentil cultivation. For long time lentil plant with improved resistance to fungal diseases has always been a breeders dream. However, these efforts were met with a limited success. In this context, plant genetic engineering and molecular breeding provide a chance to solve this problem and could broaden the gene pool in addition to conventional breeding. The productivity and the value of lentil could be greatly increased by the introduction of stably inherited traits such as pest and disease resistance, herbicide resistance and improved protein quality. These traits are not available in natural populations or near relatives of cultivated lentil varieties, but current advances in plant genetic engineering provide a potentiality for achieving these goals by genetic transformation. Moreover, molecular breeding of lentil for disease resistance genes using marker assisted selection, particularly for resistance to *Ascochyta* blight and *Anthracnose*, is underway in Australia and Canada and promising results have been obtained (Muehlbauer *et al.*, 2006). The

relative importance of lentil on the global market routine genetic engineering protocols for lentil became elaborated in the past decade.

Various environmental stress factors impose major limitations on food legume productivity. Legume yields are reduced up to 50 % due to biotic and abiotic stresses. There is an urgency to improve legume crops (commercial and desired) including lentil varieties, which are of economic importance worldwide. Particularly need is to improve these varieties and producing fungal resistant varieties. Lentil has narrow genetic base and lack of resistant gene/s of interest in available germplasm. Established tissue culture methods are a prerequisite for *in vitro* genetic manipulations, since genetic transformation entirely depends on successful *in vitro* regeneration.

In early years people have used various conventional breeding techniques to modify plants and animals to improve food production. The traditional form of genetic improvement is selective breeding, which makes it possible to select for preferred traits, such as higher yields or improved resistance of crops but this approach is very time consuming and laborious. These traditional methods of genetic modification are nowadays amended by marker assisted breeding and for haploid technologies in breeding programs (mainly cereals). The techniques of plant tissue culture have been developed as new and powerful tools for crop improvement. On the other hand biotechnology is a tool with an enormous potential for overcoming some of the inherent constraints to increase agricultural production. It adds new possibilities to accelerate plant genetic improvement. Biotechnology is aimed at re-energization of agricultural sectors, and maintaining or increasing national competitiveness.

With the recent advances in genetic engineering of plants, it is now feasible to introduce genes into crop plants that have previously been inaccessible to the conventional plant breeder, as they did not exist in the respective gene pool of the crop of interest. Genetic engineering thus has broadened the genetic variability in certain cases where the natural variability within a species is not sufficient.

Sophisticated tools of modern biotechnology depend on established transformation compatible tissue culture methods, which are up to now the bottleneck for the genetic manipulation of most leguminous crops. It is now possible to take a single gene from a plant, virus, bacteria, fungus or even animal cells and insert it into a plant to give that species a desired novel trait, such as a resistance to a destructive pest or disease.

Currently employed transformation systems require selectable marker genes encoding antibiotic or herbicide resistance, along with the gene of interest (GOI), to select transformed cells from among a large population of untransformed cells. Although rather unlikely it is of public concern that antibiotic resistance genes might be transferred from consumed transgenic food to

human intestinal bacteria, creating new antibiotic resistant strains. Others are concerned that herbicide resistance genes could be transferred to wild relatives through pollen, generating herbicide resistant 'super weeds'. Although not scientifically supported, such concerns have led to research on ways to produce selectable marker free (SMF) transgenic plants (Zhou *et al.*, 2001). Besides the above concerns, removal of marker genes offers the following research advantages. Firstly, it enables successive rounds of transformation so that useful transgenes can be stacked without crossing, secondly, retention of promoters along with selection markers which will lead to the presence of multiple copies of promoter, thereby activating signals for transcriptional gene silencing (Veluthambi *et al.,* 2003).

To date, several approaches have been developed to remove or eliminate selectable marker genes from transgenic plants such as simple microbial recombinase based systems (Hare and Chua, 2002), transposable element based systems (Yoder and Goldsbourgh, 1994), co-transformation system (Komari *et al.,* 1996), an intrachromosomal recombination (ICR) system (Zubko *et al.*, 2000), the multiautotransformation (MAT) vector system (Ebinuma *et al.*, 1997), the CLX chemically inducible system (Zuo *et al.,* 2000), homologous recombination system (Iamtham and Day, 2000) and *Cre*-lox recombination based systems (Dale and Ow, 1990). Selection and utilization of these systems differs according to removal of the selectable marker gene from the nuclear genome or from chloroplast genome (Scutt *et al.,* 2002). The main limitations of using these recombination based gene excision systems are the low efficiency of DNA recombination and requirement of time consuming crossing processes to generate SMF plants.

The basic and important part of the transformation process is to select the putative transformed explants. Our study also focuses on this matter to find an efficient method to screen out transformants. Legume transformation is difficult as the transformation frequencies are 1% or less (Chandra and Pental, 2003).

The aim of this research is to use biotechnological tools for improving one of the most important grain legume crops, the lentil, against fungal diseases by using raspberry polygalacturonase inhibitory protein gene (*Ri-pgip*). Furthermore, bringing some clarity and awareness of what advantages biotechnology can offer to the environment, health care and food security particularly in developing countries, nevertheless, the expansion of grain legume cultivation as they are superior crops from an agro-ecological point of view: they have the unique capacity of nitrogen fixation contributing to soil fertility, and enhance efficiency in agricultural rotations. From a bio-safety and acceptance point of view, it is worthwhile to notice that lentil is a self-pollinated crop limiting risk of gene flow into the wild relatives.

2. Literature Review

2.1 Legumes and Pulses - importance

The *Leguminosae* are a diverse and important family of angiosperms (Young *et al.*, 2003). This plant family is the third largest in higher plants and comprises almost 700 genera and 1800 species (Polhill and Raven, 1981). Legumes range from tiny herbs to giant trees, dominating many rainforests. Classically legumes are divided into three subfamilies, *Mimosoideae*, *Caesalpinoideae*, and *Papilionoideae* (Doyle and Luckow, 2003) where most of the cultivated and economically important legumes are found in the latter one (Zhu *et al.*, 2005). There are two major Papillionoid clades of the cultivated legume species, the so called 'Tropical' or 'Phaseoloid' legumes (including the genera *Phaseolus*, *Vigna*, *Glycine* and *Cajanus*) and the 'Temperate' or 'Galegoid' legumes (including the genera *Melilotus, Trifolium, Medicago, Pisum, Vicia, Lotus, Cicer* and *Lens*) (Young *et al.*, 2003, Zhu *et al.*, 2005). Legumes are one of the most important food crops, where the archaeological evidence suggests that the legumes have always been an important component of human diet. With the onset of agriculture, legumes became major food crops and also a source of feed for domestic animals. The legumes have always been an important component of the human diet, and still are, especially in the developing countries where pulses account for 90 % of global consumption. Economically, legumes represent the second most important crop plants after *Poaceae* (grass family), accounting for approximately 27% of the world's crop production (Graham and Vance, 2003).
The dry seeds of legumes or pulses are treated as one of the most important source of dietary protein for human or animal nutrition; some are used for edible oil. Legumes like *Alfa alfa*, clover are used as live stock feed and forage. In many developing countries of the world, grain legumes have gained much importance in view of the acute shortage in the production of animal proteins and the wide prevalence of protein malnutrition (Bressani, 1973). This makes the grain legumes to be considered as the 'Meat of the poor'. Pulses can provide B vitamins like thiamine and niacin (Gowda and Kaul, 1982), minerals like Iron 14%, calcium 2%, some potassium too and also contains carbohydrate in the form of Starch with caloric yield comparable to cereals (Aykroyd and Doughty, 1966).

2.2 Lentil (*Lens culinaris* Medik.)

2.2.1 Nomenclature

Green peas, lentils and chickpeas were common food in the roman gastronomy in ancient time. They were well known in ancient Greece as a poor man's food. A popular saying applied to the nouveau riche at the time was "he doesn't like lentils any more." The Latin word *Lens* for lentil is also descriptive in that lentil seed is shaped like a lens.

Other common names: Lentil (English), Musur (Bangla & Hindi), Linse (German), Lense (Hungarian), Adas (Arabic), Mercimek (Turkey), Lentille (French), Messer (Ethiopia), heramame (Japanese) etc.

2.2.2 Origin and Distribution

Lentils originated in the near east namely from Turkey, Syria and Iraq since the earliest evidence for the crop was 8000 - 8500 B.C. in this area. Lentil rapidly spread to Egypt, Central and Southern Europe, Mediterranean basin, Ethiopia, Afghanistan, Pakistan, India, China and later to Latin America (Cubero, 1981; Duke, 1981; Ladizinsky, 1979, Oplinger *et al.*, 1990, Vincent and Jimmerson, 2005). Now it is also being cultivated in Canada and the northwest pacific regions of the USA. It is probably the oldest of grain legumes to be domesticated (Bahl *et al.*, 1993), although it is impossible to be certain when domestication exactly began. Small lentil seeds, dating from around 10,000 BP (before present), have been found in archaeological excavations of pre-agricultural sites in Syria, but these may have been wild seeds that were gathered rather than domesticated. However, there is abundant archaeological evidence for early domestication, including a large store of lentils found in northern Israel that dates to around 8,800 BP. In some cases it is said that the oldest finds of domesticated lentil varieties in the near East date from 6000 BC. Cultivation had already spread to the Mediterranean regions and central Europe by the Neolithic age about 4000 BC. An ancestral form is Unknown. (http://en.wikipedia.org/wiki/Lentil)

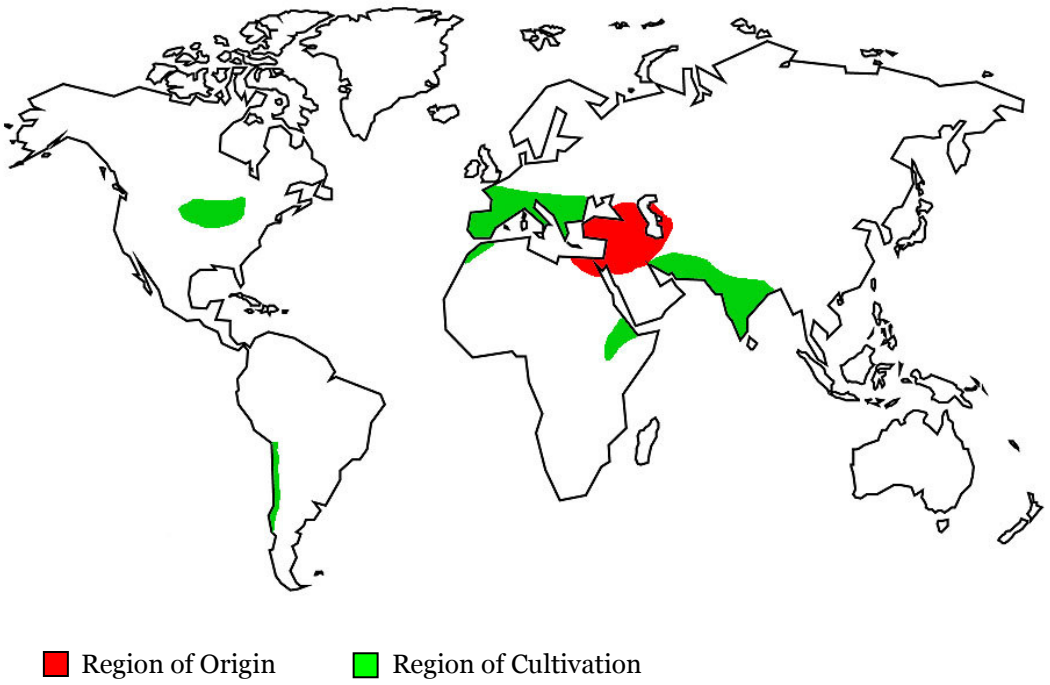


Fig. 1: World map showing centre of origin for lentils and its spread. Source: Bock D., MPI Köln

(http://www.cilr.uq.edu.au/UserImages/File/Lentil%20S14.pdf)

2.2.3 Taxonomy

Scientific name	:	*Lens culinaris* Medik.
Family	:	Leguminosae
Sub –family	:	Papilionaceae
Tribe	:	Vicieae

Chromosome 2n = 14 (Sharma *et al.*, 1995, Rubeena *et al.*, 2003). The haploid genome size of the cultivated genome is 4063 Mbp (Arumuganathan and Earle, 1991). The genus lens comprises seven taxa with four species including the cultivated type *Lens culinaris* (Furguson and Erskine 2001). *Lens orientalis* is considered to be the wild progenitor of lentil (Ladizinsky, 1993).

Lentil plants are slender, semi-erect annuals with compound leaves (4 to 7 pairs of leaflets) with a tendril at the tips. Plants normally range from 30 to 50 cm tall, the taller plants resulting from cool growing season temperatures, good moisture and good fertility. Plants can have single stems or many branches depending upon the population in the field (Oplinger *et al.*, 1990). Geographical variation pattern examination classifies cultivated forms of lentil into the two subspecies *Macrosperma* and *Microsperma* (Erskine *et al.*, 1989) on the basis of a suite of related qualitative and quantitative characters, which were relatively sensitive to environmental

conditions (Barulina, 1930). *Macrosperma* types have large pods (15-20 x 7.5-10.5 mm), generally flat, with large, flat seeds (6-8 mm dia). Cotyledons are yellow or orange. Flowers are large, white, with veins occasionally light blue. Calyx teeth are long, leaflets are large (15 -27 x 4-10 mm) and oval (length: width = 3: 3.5). Plant height ranges from 25 -75 cm, commonly grows in the Mediterranean, Africa and Asia minor.

Fig. 2: (A) Illustrated Lentil plant (b) Lentil seeds. (http://en.wikipedia.org/wiki/Lentil)

On the other hand *microsperma* types have small to medium pods (6-5 x 3.5 -7 mm) which are convex. Seeds are flattened subglobose; small to medium (3 -6 mm dia). Flowers are small and white to violet in colour with variable patterns. Height of plant varies from 15 -35 cm. usually found to grow in the South west, western and eastern areas of Asia (Gowda and Kaul, 1982). A variety of lentils exist with colours that range from yellow to red-orange to green, brown and black. The colours of the seeds also vary when removed from the pods. Seed colour may be mottled, although mottled seeds are not desirable for marketing (http://www.answers.com/topic/lentil). Lentils are cool season crops with a restricted root system which is only moderately resistant to high temperature and drought. Seeds will germinate at temperatures above freezing but best at the range of 18-21°C; temperatures above 27°C are harmful; optimum temperatures for growth and yields are around 24°C. They do not tolerate water logging or flooding. Lentils thrive on a wide range of soils from light loams and alluvial soil to black cotton soils, best on clay soils. Requires an annual precipitation of 2.8-24.3 cm and soil pH of 7.0 (Kay, 1979; Duke, 1981). (http://www.hort.purdue.edu/newcrop/cropfactsheets/lentil.html) (http://www.ag.ndsu.edu/pubs/alt-ag/lentil.htm)

2.2.4 Germplasm

The most comprehensive collection of lentil germplasm (about 7407) is maintained by ICARDA, Syria (Robertson *et al.*, 1996). The International Centre for Agriculture in Dry Areas (ICARDA) has a global mandate for research on lentil improvement. As such, ICARDA houses the world collection of *Lens*, totalling 10,509 accessions. The ICARDA collection includes 8789 accessions of cultivated lentil from 70 different countries, 1146 ICARDA breeding lines, and 574 accessions of 6 wild *Lens* taxa representing 23 countries. (http://www.icarda.org/GenerationCP/cp-1-lentil,htm). Their view in collection is to its conservation and secondly to its exploitation by breeding (Erskine *et al.*, 1988), as germplasm collections are important for all scientists interested in improving and studying any crop from genetic, physiological or pathological aspects. The Regional Plant Introduction Station located at Pullman, USA also has a collection of 2868 accessions. National programs of other countries also maintain a considerable number of germplasm accessions (Muehlbauer *et al.*, 1995; Robertson *et al.*, 1996).

2.2.5 Chemistry of Lentil

Lentil has been regarded as a clean crop, relatively free from anti-nutritional factors and low flatulence. Protein concentration in lentil range from 22 % - 35% (Oplinger *et al.*, 1990, Vincent and Jimmerson, 2005, Reddy *et al.*, 1984, El-Nahry *et al.*, 1980, El- Saied and El- Shirbeeny, 1981), 90% of it is in the cotyledons, 4% in the seed coat, 3% in the embryo, making it a cheap substitute for meat (Huisman and Poel, 1994).

Lentil seeds contain 53- 60% complex carbohydrates and 2.4 – 4.2 % minerals, (Reddy *et al.*, 1984, Oplinger *et al.*, 1990, Ofuya and Akhidue, 2005). They are an excellent source of Vitamin A, B and minerals specially Calcium, Iron, and Potassium. Folic acid is one important nutrient found in lentils (Vincent and Jimmerson, 2005). The U.S. Health Service recommends that all women of childbearing age consume 400 mg of folic acid per day. Most women do not meet this guideline. One cup of cooked lentils provides 90% of the recommended daily allowance (RDA). Lentils provide more folic acid than any other unfortified food. (http://www.pea-lentil.com/nutrition.htm#lentils)

Lentils also provide dietary fibre. It is also a source of amylase, amylopectins and high lysine (CGIAR, 2004-2005, USA Dry Pea, lentils and Chickpeas, 2006).

The nutritional value of lentil is somewhat low as it lacks in two major amino acids; methionine and cysteine (Bhatty, 1988, Vincent and Jimmerson, 2005). They are also low in sodium, fat and cholesterol.

2.2.6 Human Consumption

Lentils are often eaten as a product 'Dhal', which is a split and de-hulled seed used as a main dish, side dish or salads. Lentil seeds can also be fried or seasoned and lentil flours are used to make soups, stews, casseroles, purees, and mixed with cereals to make bread, cakes and food for infants. They are also used in gluten-free, diabetic, low salt, low calorie, low cholesterol and high fibre diets. (http://www.ampc.montana.edu/briefings/briefing61.pdf, http://www.agr.gc.ca/mad-dam/pubs/bi/pdf/bulletin18_12_2005-06- 17 e.pdf?PHPSESSID= ea148cf559ef21a8525cdc732ba0f323)

There are some Traditional Medicinal Uses of lentil too. Lentils are supposed to remedy constipation and other intestinal afflictions. "In India, lentils are poulticed onto the ulcers that follow smallpox and other slow-healing sores" (Duke, 1981).

In the 6th century, chickpeas were believed to be an aphrodisiac; while curiously enough, lentils were considered to have the opposite effect, and this was probably the reason why the lentil was included in the diet in monasteries on meatless days (Van der Maesen, 1972). (http://www.hort.purdue.edu/newcrop/cropfactsheets/lentil.html)

2.2.7 Production

The major lentil producing regions are Asia and the West Asia-North Africa region. Lentil is the most important pulse in Bangladesh and Nepal, where it significantly contributes to the diet. Farmers also grow lentils in India, Iran and Turkey. Other significant producers in the developing world include Argentina, China, Ethiopia, Morocco, Pakistan and Syria. Production is expanding due to the rising demand of an increasing population. (CGAIR, 2004-2005).

In 2004, world lentil production was over 3.8 million metric tons. Lentils are produced in over 50 different countries. India, Canada and Turkey typically combine to produce about 70 percent of total world lentil production (Fig. 3).

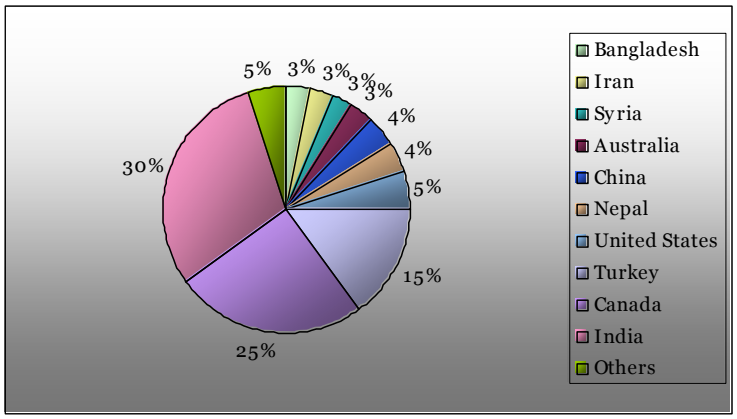


Fig. 3: World lentil production in percent by country. (Source: Agricultural Marketing Policy Center, Briefing No.61, November 2005)

World lentil production has been relatively stable over the last twelve years (Table 1). Global lentil production recently peaked at 3.8 million metric tons in 2004.

In the 2003 crop year, the three largest importing countries were Bangladesh, Pakistan, and Egypt (Table 2). Collectively, these three countries account for around 26 percent of world lentil imports. Imports of lentils are spread among many different countries. On the other side Canada is the world's leading exporter of lentils. Canada, Australia, and the United States accounted for approximately 70 percent of world lentil exports in 2003.

Year	Production in M ton
1993	2,770,230
1994	2,797,867
1995	2,855,479
1996	2,768,089
1997	2,751,809
1998	2,789,480
1999	2,890,654
2000	3,372,226
2001	3,249,845
2002	2,909,709
2003	3,104,186
2004	3,822,262

Table 1: World Lentil production 2003

Country	Rank	Import in M ton
Bangladesh	1	122,785
Pakistan	2	80,769
Egypt	3	61,177
Algeria	4	60,288
Columbia	5	52,968
Sri-Lanka	6	50,494
Spain	7	47,023
India	8	37,949
France	9	37,949
Italy	10	30,877
Others		475,625
World Total		1,057,904

Table 2: Major Lentil importers 2003

(Source: http://faostat.fao.org/site/340/DesktopDefault.aspx?PageID=340 http://www.ampc.montana.edu/briefings/briefing61.pdf)

Crops	Production M ton
Dry bean	8,167,640.00
Bean Green	2,812,832.00
Cereals total	343,663,800.00
Chickpeas	3,552,186.00
Cow peas	1,106,948.00
Ground nuts	17,331,590.00
Lupines	151,407.60
Lentils	930,213.80
Peas dry	2,049,653.00
Peas green	3,248,492.00
Pigeon peas	1,611,135.00
Pulses nes	1,107,422.00
Soybeans	46,704,130.00
Vetches	232,384.40

Table 3: World lentil production compared to other legume crops and cereals in 2005
Source: FAO statistical data, FAOSTAT database (http://faostat.fao.org/site/340/DesktopDefault.aspx?PageID=340=

Country	Production in 1000 tons
Australia	83.00
Argentina	2.00
Bangladesh	122.00
Canada	962.00
Egypt	2.62
Ecuador	1.79
Ethiopia	35.27
France	8.01
India	1,100.00
Iran	110.00
Italy	1.14
Mexico	8.60
Nepal	158.67
Pakistan	31.10
Spain	27.60
Syrian Arab Rep.	125.3
Turkey	540.00
United States of America	189.69

Table 4: Major Lentil producing countries in 2004.
Source: FAO statistical data, FAOSTAT database (http://faostat.fao.org/site/340/DesktopDefault.aspx?PageID=340)

2.2.8 Constrains of lentil production - Biotic and abiotic stress

The production of lentil is usually considerably below the established yield potential as this crop is very sensitive to particular biotic and abiotic stresses. The most serious biotic attribute constrain in lentil is the foliar diseases such as *Ascochyta* blight, rust, *Stemphylium* blight and *Botrytis* grey mold. Root rot caused by *Sclerotinia* and wilt by *Fusarium* sp. are the other two diseases responsible for yield loss. Temperature, rainfall and drought are to be listed as important abiotic constrains for lentil cultivation. These unpredictable stresses affect the cultivation of legumes in developing countries resulting in reducing cultivation despite the increased demand for legumes. Constraints affecting lentil production are divided into biotic stresses caused mainly by different micro-organisms (Table 5) and abiotic stresses.

Rust is the most important foliar disease of lentil especially in Asia, causing up to 80% to complete crop loss (Beniwal *et al.*, 1993). Ascochyta blight is another important foliar disease reported in the major lentil producing countries, including Argentina, Australia, Canada, Ethiopia, India, New Zealand, Pakistan, and The Russian Federation, and this can cost 40% yield loss (Gossen *et al.* 1986; Kaiser and Hannan, 1986; Ye *et al.,* 2002; Regan *et al.*, 2006). It is considered to be one of the major diseases of lentil in Argentina, Canada, middle- east and Indian subcontinent. *Stemphylium* blight is also prevailing in these areas with up to 80% production decrease. *Fusarium* caused wilt disease produces major economic losses in parts of South America, the Mediterranean basin and south Asia (Erskine, 1994, Bayaa *et al.,* 1995). Seedling disease root rot in lentil occurs due to invasion of *Sclerotium* (Pavgi and Upadhyay, 1967), as well as collar rot. White mold of lentil occurs from early flowering to pod setting, usually in highly productive fields with tall, dense stands of lentils. The disease is favoured by wet and cool conditions especially on lower ground where dense canopies usually develop.

(http://www.whitemoldresearch.com/HTML/lentil.cfm)

In contrast, the pea leaf weevil and the parasitic weed and to lesser extent the cyst nematode, are significant yield reducers of lentil. Lentils are very poor weed competitors.

Biotic constrains	Causal agent
Virus	*Bean yellow mosaic virus (BYMV)*
	Pea leaf roll virus (PLRV)
	Pea seed- borne Mosaic Virus (PsbMV)
	Pea Enation Mosaic virus (PEMV)
	Subterranean clover red leaf virus (SCRLV)
Fungi	
Rust	*Uromyces viciae fabae*
Stemphylium blight	*Stemphylium sarciniformis*
Ascochyta blight	*Ascochyta fabae*
Vascular wilt	*Fusarium oxyporum*
Downy mildew	*Peronospora lentis*
Anthracnose	*Colletotrichum truncatum*
Collar rot, Root rot	*Sclerotium rolfsii*
Stem rot, white mold	*Sclerotinia sclerotiorum*
Powdery mildew	*Erisyphe polygoni*
Botrytis blight	*Botrytis cinerea*
Seedling blight	*Rhizoctonia solani*
Insects	
Blue green aphid	
Pea leaf weevil	*Acyrthosiphon kondoi*
Aphids	*Sitona crinitus*
Pod borer	*Aphis craccivora & Acyrthosiphon pisum*
Seed weevil	*Etiella zinkenella*
Bruchid	*Bruchus lentis*
Red legged earth mite	*Callosobruchus cinensis*
Lucerne flea	
Nematodes	
Cyst nematode	*Heterodera ciceri*
Parasitic weeds	
Broomrape	*Orobanche spp.*
Lodging	

Table 5: Major global biotic constraints of Lentil production.
(Source: http://www.agric.gov.ab.ca/app21/rtw/search.jsp, http://www.gov.mb.ca/agriculture/crops/diseases/fac20s00.html , Regan *et al.*, 2006)

Abiotic stresses are also affecting lentil production. Among them temperature and water logging are considered most serious factors. Low temperature is a factor limiting production, but is less important than low moisture availability. High temperature is encountered by lentil in the major production regions mainly during the reproductive stage of growth. The early stages of vegetative growth are restricted by low radiation and temperature. pH plays an important role in lentil growth and nutrient availability, the optimal pH is 4.0-8.2.

2.2.9 Why apply Biotechnology in lentil improvement?

Yield loss due to diseases and pests are enormous. Unless the loss is minimized, feeding the world would be impossible. There are various ways to control diseases. Among them, resistant cultivars are paramount because they are the best way of cutting losses from disease, insects, nematodes and viruses. Besides they are cheap, dependable and the product is safe to consume (Singh, 1998). In order to achieve this objective it is important to identify the genetic needs for crop improvement as defined by plant breeders. Factors including basic physiology and genetics of pest resistance, the large number of years and locations needed to evaluate and identify stress tolerance, and the long time (in generations) needed to break up undesirable genetic linkages or to assemble desirable traits need to be examined very carefully (Cullis, 1987). Much of modern research in plant science is aimed at finding environmentally sustainable ways of controlling biotic and abiotic stresses as well as improving product quality.

Humans have been modifying their food for thousands of years. Until the 20th century, this had to be done by breeding desirable characteristics into crops. This method requires a lot of effort and is rather imprecise. That was the age of innocence. Mutagenesis and hybridization, embryo rescue *through in vitro* culture, are options to increase variation in the primary gene pool. Gene modification has enabled us to add qualities to crops that no amount of traditional breeding could. With traditional breeding methods, the available gene pool is restricted by the sexual incompatibility of interspecific and intergeneric crosses (Nisbet and web, 1990, Christou, 1994). In the 1960s scientists made huge breakthrough in their understanding of genetics and recognised that this new knowledge had the potential to revolutionise food production, creating huge benefits for the world. The green revolution in agriculture began (Chu and Higgins, 2001). This helped meet the food needs of the burgeoning human population between 1965 -1995 by producing high yielding varieties of grain. However, it was unlikely alone to assure future food security for all as the world population continues to grow in the next century. Since the result was achieved through using pesticides and continuous monocroping practices also had some unintended negative consequences like increased landlessness, disruption of social systems, loss

of beneficial farm practices and increased marginalisation of women (Chu and Higgins, 2001; Atkinson, 2006, www.biology.leeds.ac.uk/psp/publications/biotech)

After 1960s another revolutionary breakthrough went underway in 1972 when biochemist Paul Berg discovered how to join together DNA from two different organisms, creating the first recombinant DNA molecule; the beginning of recombinant DNA technology era flashed in the very next year by Stanley Cohen and Herbert Boyer as they succeeded inserting DNA from African clawed toad into *E. coli* bacterium.

Biotechnology is a recent addition to the techniques of plant improvement by genetic approaches. It can be defined as consisting two distinct technologies, firstly is the 'marker technology' in which genetic markers (DNA fragments) are used in marker-assisted-selection (MAS) to identify and expedite the combining of existing desirable characters within new plant line. Secondly, is the 'gene technology', where desirable genes are reconstructed by recombinant DNA technology methods and transferred into plants (Chu and Higgins, 2001). Plants derived using marker technology are not considered as genetically modified but the ones derived using gene technology are termed as genetically engineered or modified organisms (GMOs).

Biotechnology has opened a new horizon and ways to control different stresses and to improve crop quality and quantity by enabling rapid transfer of specific genes from different organisms (e.g. unrelated species, wild relatives, or completely different organisms like bacteria, fungi, virus or even human) to overcome the crossing barriers and resulting in extension of the variability and gene pools which can be integrated in breeding programs much faster than with normal breeding strategies (Hassan, 2006).

Biotechnology can be exploited for consumers benefit, particularly in the developing countries. The quality of food and food plants can be modified and optimized to meet the nutritional and health needs of at-risk and compromised populations prevalent in most of the developing countries. High rates of malnutrition, infectious diseases as well as diet-related diseases such as diabetes and hypertension are prevalent in many developing countries (Niba, 2003). The technology improves the poor communities' life, Golden rice for example. Rice lacks in vitamin A, has now been modified with genes form daffodil and a bacterium so that it can produce its own carotene (vitamin A). Rice could never have been crossed with daffodil to do this by traditional methods. Golden rice has the potential of preventing 3 million deaths caused by vitamin A deficient children who also easily catch diarrhoea and measles. It will also prevent 5 million children from falling victim to xeropthalmia each year (Siraj, 2001).
In a recent survey scientific experts around the world indicated that the top biotechnological need for developing countries was for diagnosis of infectious disease, other needs such as

vaccines and increased nutrient content of food crops as well as combinatorial chemistry for drug discovery etc (Daar *et al.,* 2002).

This technology has its own role to play in the world business and economy. A first example was the Flavr Savr tomato, the first GM food that appeared on the market in 1994, it was modified genetically to keep it firm for longer. The herbicide tolerant crops are another example. Soybean, Corn, Cotton are the most successful GM crops in the world. Other examples are the 'toxic crops' that produces their own pesticides, the BT maize and BT cotton, modified with a gene from *Bacillus thuringiensis* to kill pests like corn borer.

Since initial commercialization in 1996, global planted area of biotech crops has soared by more than fifty-fold from 4.2 million acres in six countries to 222 million acres in 21 countries in 2005. The increase was 9 million hectares or 22 million acres, equivalent to an annual growth rate of 11% in 2005. The 8.5 million farmers planting biotech crops in 2005 also marked a significant milestone as the 1 billionth cumulative acre, or 400 millionth hectare, was planted. (James, 2005). (http://www.isaaa.org/kc/bin/briefs34/pk/index.htm)

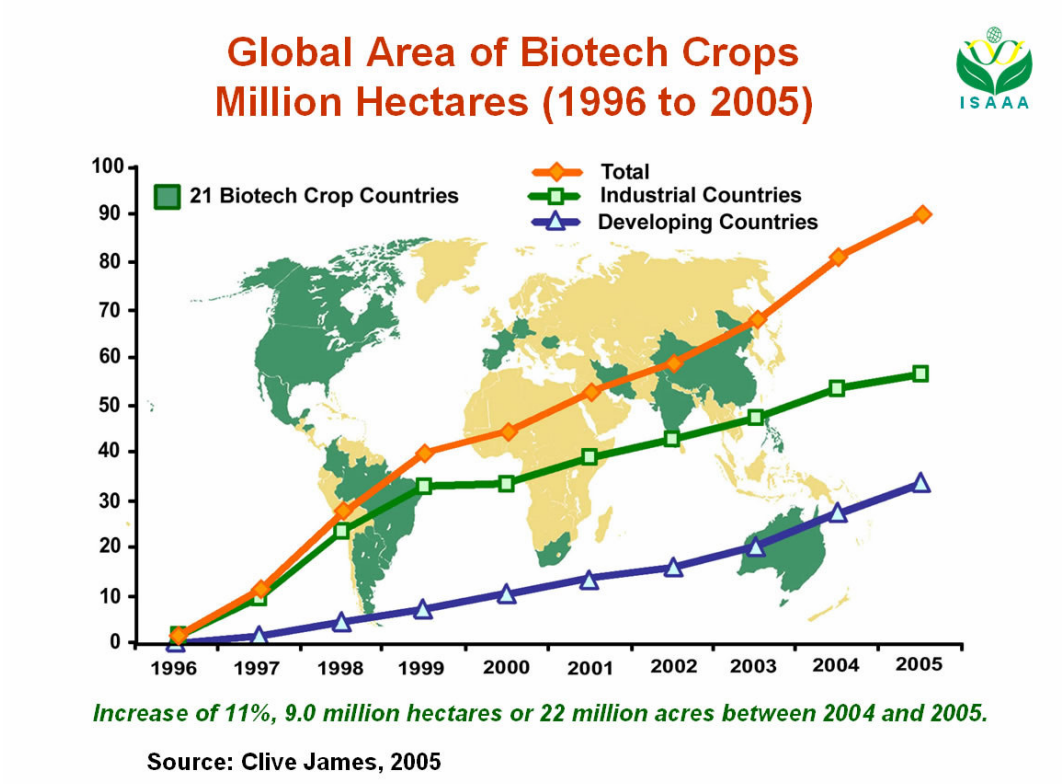


Fig. 4 : Global area of Biotech crops.
(http://www.isaaa.org/kc/CBTNews/press_release/images/briefs34/figures/hectares/figure%20cover_hectares.jpg)

2.2.10 Strategies for the development of fungus-resistant transgenic plants

Scientists over the world have achieved in developing different approaches to develop fungal resistance in plants. The adopted strategies can be basically classified into two categories (Grover and Gowthaman, 2003), namely -

- Production of transgenic plants with antifungal molecules like proteins and toxins
- Generation of a hypersensitive response through R genes or by manipulating genes of SAR pathway

Plants already have defence systems which involve pathogen-related proteins, e.g. polygalacturonase-inhibiting proteins (Faize *et al.*, 2003; Agüero *et al.*, 2005), chitinase (Legrand *et al.*, 1987; Shinshi *et al.*, 1990; Yamamoto *et al.*, 2000), stilbenes (Wiese *et al.*, 1994; Hain *et al.*, 1993), or β-1,3-glucanase (Kombrink *et al.*, 1988).

A set of protein, that are induced by pathogen infection, wounding, fungal cell wall elicitors, Ethylene, UV light, heavy metals etc. PR protein group confer a group of 5 families (PR1 –PR5) based on primary structure, serological relatedness, enzymatic and biological activities (Agrios, 1997; Grover and Gowthaman, 2003). All members of these families show antifungal *in vitro* activity by inhibiting hyphal and fungal growth as reported by many scientists (Broglie *et al.,* 1991, Asao *et al.*, 1997; Bolar *et al.*, 2000; Rajasekaran *et al.*, 2000; Boller, 1993). PR proteins are induced during hypersensitive responses (HR) and also during systemic acquired resistance (SAR) and therefore are thought to have a role in natural defence or resistance of plants against pathogens.

RIP-Proteins having N- glycosidase activity removing an adenine residue from 28s rRNA cause inhibition of protein elongation. RIPs inactivates foreign ribosomes of distantly related species and of other Eukaryotes including fungi (Logemann *et al.,* 1992). A number of small cysteine rich proteins are forming a separate group of antifungal polypeptides; e.g. Chitin binding proteins, Plant defensins & Thionins. Lipid transfer proteins (LTPs) stimulate the transfer of a broad range of lipids through membranes. 2s storage proteins have dual roles – storage protein and plant defence; they can inhibit the growth of pathogenic fungi (Terras *et al.,* 1995). Polygalacturonase inhibitor proteins (*PGIP*s) are proteinaceous inhibitors of fungal polygalacturonase which enable pathogen infection by facilitating host cell wall degradation and *PGIP*s interference with this process (Powell *et al.,* 2000). Along with the mentioned systems there are also the antiviral proteins (Poke weed antivirus protein) which if constitutively

expressing in high level conferrers resistance in the host plant against different viruses (Wang *et al.*, 1998) and the non-plant antifungal proteins (Faize *et al.*, 2003; Gao, 2000) are playing role against fungal activity. Cell wall degrading enzymes (Jach *et al.*, 1995), double stranded RNA viruses encoding antifungal proteinaceous killer toxins (Park *et al.*, 1996), bacterial peptide (Mitsuhara *et al.,* 2000), egg white lysozyme (HEWL) (Trudel *et al.*, 1995), synthetic gene encoding for chimeric cationic antimicrobial peptide (CAP) (Osusky *et al.,* 2000) and synthetic cationic peptides (Ali and Reddy, 2000) are also applied important non plant antifungal proteins.

Phytoalexins are low molecular weight antimicrobial secondary products also used in the defence system against fungi (Hain *et al.,* 1993).

Since there is a possibility of the fungal invaders to overcome the plant defence system by the PR, phytoalexins, toxins etc some other approaches in defending from the fungal invasion are in process like resistance genes from plants (Takken and Joosten, 2000), broad spectrum disease resistance using SAR (systemic acquired resistance) (Clarke *et al.*, 1998) or induced cell death by Oxidative burst – H_2O_2 triggering production of phytoalexins, PR proteins, other HR related process (Lamb and Dixon, 1997).

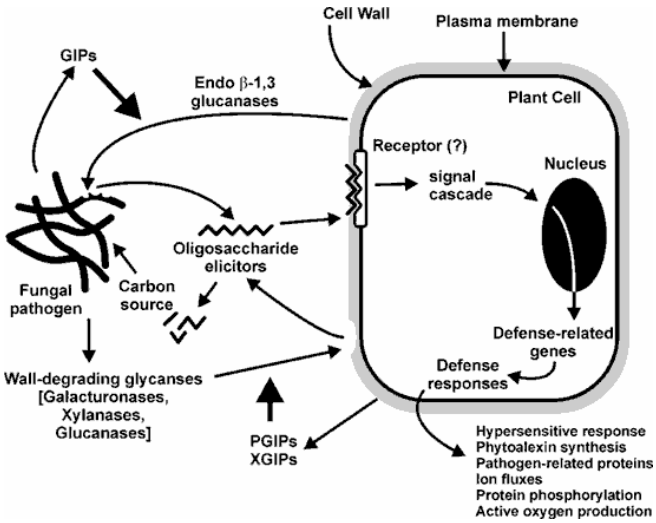


Fig. 5: Higher plants defence response. Source: (http://www.ccrc.uga.edu/~mao/plapath/PPtext.htm)

2.2.11 Polygalacturonase inhibitory proteins (*PGIP*s)

Plant cell walls are one of the barriers against pathogenic fungi. A majority of fungi needs to break this barrier to gain access to the plant cells and therefore secrets endopolygalacturonases (PGs). These enzymes are capable of degrading plant cell wall polymers and thus result in cell

maceration. Fungal PGs also release oligogalacturonide (OG) fragments from the plant cell walls, which are the elicitors of a variety of defence responses. These 'OGs' are produced by action of PGs if the enzyme action is controlled by specific protein inhibitors called *PGIP*s (PG inhibiting proteins).

Polygalaturonase-inhibiting proteins (*PGIP*s) are extra cellular plant proteins capable of inhibiting fungal PGs. These proteins are localized in cell walls of many plants and have the potential for limiting fungal colonization by acting as both inhibitors and regulators of PG activity and by favouring the release of OGs.

The occurrence of PGIPs has been reported in a variety of dicotyledonous plants and in the pectin rich monocotyledonous plants. Plants have evolved different PGIPs with specific recognition capabilities against the many PGs produced by phyto-pathogenic fungi as these PGs exhibit a variety of isoenzymetic forms, differing in terms of stability, specific activity, pH optimum, substrate preference and types of oligosaccharide released(De Lorenzo *et al.*, 1997). It is said that PGIPs are typically effective against different fungal PGs but ineffective against other pectic enzymes from microbial or plant origin (Cervone *et al.*, 1990). PGIPs from different plant sources differ in their activities and also single plant source been seen to inhibit PGs from different fungi or different PGs from same fungus (Cook *et al.*, 1999).

The first gene encoding a PGIP was cloned from *Phaseolus vulgaris* (Toubart *et al.*, 1992). Now these genes have been cloned from many plant species where they exist as gene families. In raspberry, tomato, pear and apple there are at least two PGIP related genes present. A small family is also present in grape (Ramanathan *et al.*, 1997). Most of the characterized PGIP genes are not interrupted by introns except in *Arabidopsis* and raspberry and interestingly the position of the introns is maintained in both (Ramanathan *et al.*, 1997).
Typically *PGIP* genes code for protein products comprising a signal peptide for translocation into the endoplasmic reticulum and a mature polypeptide of 300-315 amino acids with several potential glycosylation sites. The mature *PGIP* is characterized by the presence of 10 repeats, each derived from modifications of a 24 amino acid leucine rich peptide. The Leucine rich repeat (LRR) element matches the extracytoplasmic consensus GxIPxxLxxLxxLxxLxLxxNxLx (here, 'I' indicates I or L, 'L' indicates L, I, V, F or M and 'x' indicates any amino acid), which is also found in several resistance (R) genes that participates in gene to gene resistance that is PGIPs share striking similarities in terms of structure and specificity with the R gene products it pointing to an important role in defence against pathogens. The recognition ability of *PGIP*s resides in their LRR structure where solvent-exposed residues in the ß-strand/ß-turn motifs of the LRRs are determinants of specificity.

In a single plant species, different members of the *PGIP* family may encode *PGIP*S with nearly identical biochemical characteristics. To date, only plants have been successfully used to express functional *PGIP*s, either stably through genetic transformation or transiently through infection (Desiderio *et al.,* 1997). On the basis of amino acid sequence identity, mature *PGIP*s can be grouped into two clusters, one including legume *PGIP*s and the other one with the rest. Sequence similarity among *PGIP*s of different species belonging to the same plant family is sometimes very high though the species may be phylogenetically rather distant. Differences among *PGIP*s mainly depend on substitutions and insertion a/deletion of a few amino acids, indicating that duplication and point mutations are the major driving force for the evolution of *PGIP* families (De Lorenzo *et al.,* 2001).

The expression of *PGIP*s is spatially and temporally regulated during development and in response to several stress stimuli like elicitors such as OGs and fungal glucans, wounding or treatment with salicylic acid (Bergmann *et al.,* 1994). For example, in bean low level activity detected in all tissues of growing plants and high activity in the transition zones (Salvi *et al.*, 1990). *PGIP* transcripts are seen to be induced by wounding and pathogen infection in soybean (Favaron *et al.*, 2000). Levels of *PGIP* transcripts vary in different plant species. It is much less in green seedlings than in etiolated seedlings in *Phaseolus* (Devoto *et al.,* 1997) or in flowers than fruit of pear (Stotz *et al.*, 1993). Transcript levels correlate with the activity levels except for the constant levels found in raspberry during maturation (Ramanathan *et al.,* 1997). In *Arabidopsis thaliana*, transgenic plants expressing an antisense *AtPGIP1* gene have reduced At*PGIP*1 inhibitory activity and were more susceptible to *B. cinerea* infection noting that *PGIP* contributes to basal resistance to pathogen *B. cinerea* and strongly supports the vision that this protein plays a role in *Arabidopsis* innate immunity (Ferrari *et al.*, 2006).

2.2.12 *Agrobacterium*-mediated genetic transformation of lentil

Advocates have argued that biotechnology will lead the next revolution in agricultural production, and substantial economic resources are being used to bring this vision about. Biotechnology industry has used the issue of world hunger as a cornerstone (White *et al.*, 2004) and legumes are playing one of the main roles to minimize world hunger. Genetic transformation has potential impacts for crop improvement through alleviation of specific production constraints.

The key transformation events in grain legumes can mainly be focused on *Agrobacterium*-mediated transformation, Biolistics for gene delivery, Electroporation and /or Polyethylene glycol (PEG) treatment.

Stable transformation has been reported for a number of legumes using common indirect transformation method with *Agrobacterium tumefaciens* or *A. rhizogenes*, otherwise direct gene transfer method like particle bombardment (Gulati, 2002, Masood *et al.*, 1996, Öktem *et al.*, 1999) and electroporation of protoplasts (Christou, 1994, Atkins and smith 1997), PEG-mediated gene transfer (Böhmer *et al.*, 1995; Maccarrone *et al.*, 1995). Some alternative methods which showed potentiality have also been used on legumes such as *in planta* transformation (Chee and Slightom, 1995) or electroporation of apical meristems (Chowrira *et al.*, 1995, 1996). The advantage with these methods is that they are side stepping the tissue culture part of the whole transformation work as an efficient regeneration system is must for any transformation program. Since legumes are recalcitrant in nature (Collén and Jarl, 1999, Nisbet and Webb, 1990, de Kathen and Jacobsen, 1990, Fratini and Ruiz, 2003) this possibility of step elimination is very important for legume crops improvement.

The production of transgenic plants using *Agrobacterium tumefaciens* as a gene vector was limited to dicotyledons usually, for examples pea (Schroeder *et al.*, 1993, de Kathen and Jacobsen, 1990) soybean (Hinchee *et al.*, 1988), *Alfa alfa* (Deak *et al.*, 1986), white clover (White and Greenwood, 1987) , subclover (Khan *et al.*, 1994), chickpea (Kiesecker, 2000) and cowpea (Ikea *et al.,* 2003) etc but it has been now in use for monocots too, such as rice (Upadhaya *et al.*, 2000), barley (Matthews *et al.,* 2001), wheat (Bhalla, 2006), maize (Escudero *et al.*, 1996) etc.

It may be mentioned here that though Lentil (*Lens culinaris* Medik.) is a source of dietary protein and is an important pulse crop yet only a few reports are available regarding regeneration and transformation. Lentil transformation by *Agrobacterium* has been reported with limited success (Warkentin and McHughen, 1993). Moreover, it is a fact that the type of strain used in transformation work has its own influence on transformation efficiency (Grant *et al.*, 2003).

Lentil is susceptible to transformation by virulent strains of *Agrobacterium tumefaciens* (Warkentin and McHughen, 1991; Khawar and Özcan, 2002). One of the earliest reports of lentil transformation showed that four diverse strains of *Agrobacterium tumefaciens* were capable of inducing tumours at a high frequency on inoculated stems of lentil (*Lens culinaris* Medik. cultivar Laird) *in vivo*, and on excised shoot apices *in vitro*. Tumour formation and opine production are indicative of plant cell transformation (Warkentin and McHughen, 1991).

As an initial step in the development of transgenic plants, it is useful to demonstrate that tissues of that species are capable of expressing a transferred reporter gene. Transient assays allow the monitoring of gene expression shortly after transformation (Davey *et al.,* 1989). Lentil (*Lens culinaris* Medik.) shoot apex, epicotyl, and root explants were capable of expressing an intron-

containing beta-glucuronidase (GUS) gene after inoculation with the disarmed *Agrobacterium* strain. Expression occurred at all wound sites on these explants except at the end of the root explants proximal to the cotyledonary node (Warkentin and McHughen, 1992).

Lentil seedling root protoplasts were tested for transient expression system through electroporation and PEG treatment (Maccarrone *et al.*, 1995). Transient GUS activity has been detected in lentil protoplasts and cotyledonary nodes, following delivery of the genes via liposomes (Maccarrone *et al.*, 1992) or particle bombardment (Öktem *et al.*, 1999). GUS expression has also been observed after inoculation of longitudinally sliced embryogenic axes of lentil with different *Agrobacterium* strains (Lurquin *et al.*, 1998). No transgenic lentil plants were reported in any of these studies. Lentil cotyledonary nodes are some of the most regenerative tissues in legumes. Attempts to transform them by vacuum filtration have been limitedly successful. The first report of a vacuum infiltration *Agrobacterium* mediated transient expression system on lentil cotyledonary nodes was by Mahmoudian *et al.*, in 2002. The effect of micro-wounding by particle bombardment, wounding by sonication, macro-wounding by needle and the coupling of vacuum infiltration to *Agrobacterium*-mediated transformation, on the efficiency of transient GUS expression of cotyledonary node tissues were also investigated in order to optimization of an *Agrobacterium* infiltration based transformation system for lentil (Ufuk *et al.*, poster presentation, http://abstracts.aspb.org/pb2006/public/P46/P46047.html).

There are advantages and disadvantages linked to all the methods. Even in conventional breeding one can neither foresee nor control what the physiological impact of the genes might be, given the genetic background of the host plant. *Agrobacterium* mediated transformation has some advantages in comparison to other systems, for example its respective simplicity without need for highly sophisticated equipments, predictable integration patterns of the transgene, possibility to transfer large fragments of T-DNA and relatively stable transformation events. The disadvantage of it is, not all species are susceptible to *Agrobacterium* infection.

2.2.13 Regeneration

Plant tissue regenerates *in vitro* through two pathways, namely organogenesis or embryogenesis; while in organogenesis pathway shoot buds are formed by inducing meristematic activity in some cells, and somatic embryogenesis involves differentiation process from a single cell or a cluster of cells to form somatic embryos that follow the pathway of zygotic embryos (Chandra and Pental, 2003).

Although many legumes have been regenerated using tissue culture techniques, very few

efficient and reproducible regeneration protocols are presently available to be used in transformation experiments. This seriously impeded the application of gene transfer to improve leguminous crops. Various explants and plant growth hormones have been used for developing efficient regeneration systems for grain legumes. According to various reports 'reproducible' regeneration protocols thought to be possible using axillary meristem from cotyledonary nodes of chickpea (Jayanand *et al.*, 2002), soybean (Wright *et al.*, 1986), bean (McClean and Grafton, 1989) and pea (Schroeder *et al.*, 1995, Jackson and Hobbs, 1990). De Kathen and Jacobsen (1990) used epicotyl segments and node explants from etiolated seedlings of pea while Schroeder *et al.* (1993) developed transgenic peas through organogenesis using longitudinal slices embryogenic axis of immature seeds. In the following years, Grant *et al.* (1995) developed a transformation system for four pea cultivars using immature cotyledon explants. Complete plantlets were regenerated via leaf derived callus in pea using Picloram (Jacobsen and Kysely, 1984), or from immature zygotic embryos or from shoot apices using 2, 4-D or Picloram (Lehminger-Mertens and Jacobsen, 1989; Kysely and Jacobsen, 1990). Regeneration of plantlets via somatic embryogenesis from leaf explants in chickpea also but the embryos were prone to re-callusing (Dineshkumar *et al.,* 1995). *In vitro* organogenesis of *Vigna radiata* from hypocotyl and cotyledon explants raised adventitious shoots on MS medium containing BA, NAA and Coconut water (Amutha *et al.*, 2002) while in *Vigna mungo* cotyledonary node explants showed BA is essential for multiple shoot induction (Saini *et al.*, 2003). An efficient regeneration protocol of *Cajanus* was reported based on leaf explants on BA and Kn supplemented MS media followed by subculturing the regenerated shoot buds on GA3 containing medium (Dayal *et al.*, 2003). Embryo axes explants can develop direct shoot organogenesis in *Vigna subterranea* on media containing BAP or TDZ and BAP, NAA (Lacroix *et al.*, 2003).

As mentioned before regeneration is the critical step for any transformation success in grain legumes. Different techniques are being used for regeneration of lentil through organogenesis via callus or direct shoot regeneration. *In vitro* culture of lentil has proved difficult though the techniques have been progressively improved in the last 20 years (Ye *et al.*, 2002). The first report of *in vitro* regeneration from lentil meristem tip was by Bajaj and Dhanju (1979). Regeneration was also obtained from callus cells using hypocotyl and epicotyl explants (Williams and McHughen, 1986). Protoplasts from epicotyls were used to form callus for organogenesis of lentil (Rozwadowski *et al.*, 1990). Using callus induced embryonic axes via somatic embryogenesis whole plants were obtained by Saxena and King (1987). Multiple shoots were regenerated from shoot tips, first nodes and first pairs of leaves in BA or BA with NAA supplemented media (Polanco *et al.,* 1988). Nodal segments, shoot tips or callus, all these

explants from lentil can produce plants and presence of kinetin influence multiple shoot formation from nodal segment and shoot tip explants (Williams and McHughen, 1986, Singh and Raghuvanshi, 1989). Thidiazuron (TDZ) also had a greater effect on multiple shoot regeneration of lentil (Murthy *et al,* 1998, Saxena and Malik, 1992). High frequency shoot regeneration was also possible from intact seedlings of pea, chickpea and lentil using TDZ (Malik and Saxena, 1992). Using only BA showed higher frequency towards multiple shoot formation from cotyledonary explants (Warkentin and McHughen, 1993, Mallick and Rashid, 1989, Gulati *et al.*, 2001). Bisected (Halbach, 1998) or decapitated embryos of lentil were found forming multiple shoots BAP, Kn, and GA_3 supplemented MS medium; and addition of tyrosine increases the efficiency (Sarker *et al.,* 2003).

Pulse crops have long been considered to be recalcitrant to cell and tissue culture, with lentil being one of the most difficult legumes to regenerate whole plants due to problems in root induction (Fratini and Ruiz, 2003). The induction of root morphogenesis to obtain whole plants from legumes has been conventionally approached by means of using different auxins at different concentrations. Rooting in lentil shoots on half or full strength MS medium and B5 vitamins supplemented with 2.5µM NAA. 40 -50% success was obtained by Malik and Saxena (1992). They also mentioned that shoots excised from *in vitro* cultures with TDZ were difficult to root. Shoots which developed in presence of BAP could be rooted up to 4.6 – 39.9% in media containing IBA (Polanco and Ruiz, 1997) but faced inhibitory effect of BAP, Kn towards rooting of lentil shoots *in vitro*. Lentil shoots regenerated from media with Kn and 2,4-D induces root in hormone free medium was reported by Singh and Raghuvanshi (1989).

An inverted orientation of the nodal explant derived shoots on MS medium supplemented with 5µM IAA and 1µM Kn gave raise to 95.35% roots in lentil (Fratini and Ruiz, 2003). NAA was found to be more effective than IBA in root formation in lentil while using lentil seeds as explants (Ye *et al.*, 2002) and also in *Vigna subterranea* (Lacroix *et al.*, 2003). Use of IBA worked efficiently for rooting in case of *Vigna radiata* shoots derived from either hypocotyls or cotyledons (Amutha *et al.*, 2003), also in *Cajanus cajan* (Dayal *et al.*, 2003). Filter-paper bridges immersed in liquid rooting medium containing IBA helped in producing roots in chickpea (Jayanand *et al.,* 2003).

Micro-grafting is considered a better option for rooting in *Lens* than using phytohormones (Hassan, 2001, Gulati *et al.,* 2001) as also in *Cicer arietinum* (Krishnamurthy *et al.*, 2000) , *Phaseolus acutifolium* (Clereq *et al.*, 2002), *Pisum sativum* (Bean *et al.*, 1997), *Vicia faba* (Pickardt *et al.,* 1995).

2.2.14 Selectable Markers

Selectable marker genes are required to ensure the efficient genetic modification of plants. Two types of selectable marker genes are commonly used during gene cloning and plant transformation: (1) selectable marker genes which are integrated into the recipient genome along with the GOI, allowing survival of transformed plant cells against the large background of non transformed cells, (usually antibiotic or herbicide resistant genes) or (2) bacterial expressed selectable marker genes, these are plasmid borne markers encoding resistance to antibiotics allowing the selection and maintenance of transformed bacterial cells against non transformed cells (Goldsbrough, 2001).

Approximately 25 selectable marker genes so far have been in use for the plant transformation work. These are mostly conferring resistance to antibiotics, herbicides or metabolic inhibitors (table 6).

Gene	Gene source	Gene product	Selective agent
aadA	*Shigella flexneri*	Aminoglycoside-3-adenyltransferase	Streptomycin, spectinomycin
accC3/accC4	*Serratia marcescens, Klebsiella pneumoniae*	Gentamycin-3-N-acetyltransferase	Gentamycin
AK	*Escherichia coli*	Aspartate kinase	High concentration lysine and threonine
als	*Arabidopsis thaliana, Nicotiana tabacum*	Acetolactate synthase	Sulfonyl ureas, imidazolinones, thiazolopyrimidines
BADH	*Spinacea oleracea*	Betaine aldehyde dehydrogenase	Betaine aldehyde
bar	*Streptomyces hygroscopicus*	Phosphinothricin acetyltransferase	Glufosinolate, L-phosphinthrin, bialaphos
bla	*Escherichia coli*	b-Lactamase	Penicillin, ampicillin
Ble	*E. coli* TN5, *Streptoalloteichus hindustanus*	Bleomycin resistance protein	Bleomycin, phleomycin
bxn	*Klebsiella pneumoniae var. iozaenae*	Bromoxynil nitrilase	Bromoxynil
cat	Bacteriophage P1 Cm R	Chloramphenicol acetyltransferase	Chloramphenicol
dhfr	Plasmid R67	Dihydrofolate reductase	Methotrexate

Continued...

Gene	Gene source	Gene product	Selective agent
DHPS	*Escherichia coli*	Dihydrodipicolinate sythase	S-aminethyl L-cysteine
epsps/aroA	*Agrobacterium* CP4, maize, *Petunia*	5-Enoylpyruvate shikimate -3 -phosphate	Glyphosate
Gfp	*Aequorea victoria*	Fluorescent chromophore	
hpt	*Escherichia coli*	Hygromycin phosphotransferase	Hygomycin B
manA	*Escherichia coli*	Phosphomannose isomerase	Mannose-6-phosphate
nptII	*Escherichia coli* Tn5	Neomycin phosphtransferase II	Kanamycin, neomycin, geneticin (G418), paromommycin, amikacin
pat	*Streptomyces viridochromogenes*	Phosphinothricin acetyltransferase	Glufosinolate, L-phosphinthrin, bialaphos
SPT	*Escherichia coli* Tn5	Streptomycin phosphotransferase	Streptomycin
sul	Plasmid R46	Dihydropteroate synthase	Sulfonamide
TDC	*Catharanthus roseus*	Tryptophan decarboxylase	4-Methyltryptophan
tfdA	*Alcaligenes eutrophus*	2,4-D Monooxygenase	2,4-Dichlorophenoxyacetic acid
uidA/GUS	*Escherichia coli*	β-Glucuronidase	Cytokinin glucuronides
xylA	*Thermoanaerobacterium thermosulfurogenes*	Xylulose isomerase	D-Xylose

Table 6: Marker genes and selective agents used in plant transformation (Scutt *et al.*, 2002; Hare and Chua, 2002; Jaiwal *et al.*, 2002).

2.2.15 Marker free Transformation

Marker genes so far have been considered of being indispensable for identifying the rare events that have taken up foreign DNA. Whether or not the removal of marker genes from plant genomes has been a controversial topic, the benefits are worth considering (Ow, 2001). As mentioned before,

following transformation the continued presence of marker genes in the genetically modified plants usually becomes unnecessary or may also be undesirable though these are conditionally dominant genes that confer ability for the transformed plant to survive in the applied selective agents that are toxic to plant cells or inhibitory to plant growth.

The presence of marker genes encoding antibiotic or herbicide resistances in genetically modified plants poses a number of problems for example; herbicide resistance genes could escape to wild relatives of the crop through transfer of pollen, potentially leading to the spread of herbicide resistance in the wild plant population. The presence of antibiotic resistance markers in the transgenic crops meant for human and animal consumption may cause horizontal transfer of the gene to micro-organisms of the gut flora of man and animals and thus leading to spread of antibiotic resistance in the pathogenic micro-organisms (Scutt *et al.*, 2002). Extensive studies have failed to detect a quantifiable risk of this occurrence. In addition to the unlikely environmental and health concerns, there are also practical reasons for the removal of unnecessary marker genes. Firstly, it allows the same marker to use for the sequential addition of further transgenes, secondly, there is a greater possibility of instability of transgene expression if several homologous marker gene copies are present in the same plant. Presence of multiple copies of marker genes poses the possibility of silencing the required transgene through homology dependent gene silencing mechanisms (Scutt *et al.*, 2002).

Various techniques are under development for removal of unwanted marker genes but leaving required transgenes in place. (Ebinuma *et al.,* 2001, Goldsbrough, 2001, Scutt, *et al.*, 2002, Hare and Chua, 2002, Jaiwal *et al.,* 2002, Afolabi *et al.*, 2004, Sun and Zuo, 2003). These techniques can be divided into the following categories (1) Simple microbial recombinase based systems (Hare and Chua, 2002), (2) Transposable element based systems (Goldsbourgh *et al.*, 1993), (3) Co-transformation systems (Komari *et al.,* 1996, De Framond *et al.*, 1986), (4) an intrachromosomal recombination (ICR) system (Zubko *et al.*, 2000), (5) the multiauto-transformation (MAT) vector system (Ebinuma *et al.*, 1997, Endo *et al.,* 2002, Sugita *et al.*, 2000), (6) the CLX chemically inducible system (Zuo *et al.,* 2000), (7) homologous recombination system (Iamtham and Day, 2000, Zubko *et al.*, 2000) and (8) *Cre*-lox recombination based systems (Srivastava and Ow, 2004, Dale and Ow, 1990, Yuan *et al.*, 2004). These systems differ according to removal of the selectable marker gene from the nuclear genome or from Chloroplast genome (Scutt *et al.,* 2002).

Techniques based on DNA recombination and *Agrobacterium* mediated transformation co-transformation with two binary vectors in a single or two different *Agrobacterium* strains or with super binary vectors carrying two sets of T-DNA border sequences (twin T-DNA vectors), have been employed to produce SMFs (Selectable marker free) (Lu *et al.*, 2001). By introducing additional T-

DNA borders into a binary plasmid used in *Agrobacterium*-mediated plant transformation, previous studies have demonstrated that the marker gene and the gene of interest (GOI) can be carried by independent T-strands, which sometimes integrate in unlinked loci in the plant genome. This allows the recovery of marker-free transgenic plants through genetic segregation in the next generation.

Among the different approaches towards selectable marker free (SMF) transformant production, co-transformation has widely been used by various authors. A large number of tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) transformants are produced through co- transformation by *Agrobacterium* strain LBA4404 with vectors carrying two separate T-DNAs, one with a drug resistance selection marker and the other containing a GUS gene. Segregation of the transformants rendered plants being free from selection markers (Komari *et al.,* 1996). Marker free tobacco plants were obtained through co-transformation using a negative selectable marker gene *codA* lying next to *npt*II in pNC vector while the second vector pHG contained GUS (Park *et al.,* 2004). In a same manner selectable marker free (SMF) rapeseed (*Brassica napus*) and tobacco were obtained by using different plasmid vectors (Daley *et al.*, 1998), and also barley (Matthews *et al.,* 2001). Transformation of *Vigna mungo* was carried out with the *Cre* –lox system containing to produce marker free salt tolerant transgenics (Sarin *et al.,* 2004). A new *cre*-lox system using a single vector was reported to be efficient in producing SMF tobacco (Yuan *et al.*, 2004). The MAT-vector system is the only tool that has been successfully applied in practical plant species, such as rice (Endo *et al.,* 2002) and hybrid aspen (Ebinuma *et al.*, 1997), in addition to tobacco and *Arabidopsis*.

Plastid DNA recombination and cytoplasmic sorting was exploited to remove *aadA* (Aminoglycoside-3-adenyltransferase conferring resistance to spectinomycin and streptomycin) from transplastomic tobacco plants where *aadA* was flanked by *bar* and *uidA* genes (Iamtham and Day, 2000). Tobacco chloroplasts were transformed to reconstitute wild type pigmentation in combination with plastid transformation vectors, preventing stable integration of the marker gene leading to marker free transformants in the first generation (Klaus *et al.*, 2004). SMF approach has also been adopted for ornamentals and woody plants (Matsunaga, *et al.*, 2002).

By repositioning the selectable marker gene in the backbone and leaving only the GOI in the T-DNA region, a regular two-border binary plasmid was able to generate marker-free transgenic maize plants more efficiently than a conventional single binary plasmid with multiple T-DNA borders (Huang *et al.,* 2001). These results also provide evidence that both the right and left borders can initiate and terminate T-strands. Such non-canonical initiation and termination of T-strands may be the basis for the elevated frequencies of co-transformation and unlinked insertions. Using double right border (DRB) vector system marker free transgenic rice was recovered (Lu *et al.,* 2001).

3. OBJECTIVES OF THE STUDY

This research proposes to improve the second most important grain legume crop for human nutrition, lentil, against fungal diseases using PGIP gene with the need to focus on what advantages biotechnology can offer to the environment, health care and food security particularly in developing countries.

The ultimate goal of this study is to enhance the resistance to fungal disease in lentil through the expression of *Ri -pgip* gene from raspberry. The study focuses on the following objectives:

1. Develop an efficient *in vitro* regeneration system of lentil compatible for *Agrobacterium* mediated genetic transformation
2. Protocol for *Agrobacterium* mediated genetic transformation
    - Transformation of lentil via *Agrobacterium*- mediated system.
    - Molecular characterization of the transformants.
    - Evaluation of the genetically modified plants for their fungal resistance
3. A marker free transformation system
    - Cloning of *Ri-pgip* gene into a binary vector.

To achieve these aims, the plant binary vector of pGreenII series was used and the *Ri-pgip* gene was driven by a 35S cauliflower mosaic virus (*CaMV*) constitutive promoter. Subsequently the constructs would be transformed to *Agrobacterium tumefaciens* strain EHA105. A modified method adapted from Schroeder *et al.* (1993) was used for lentil transformation (*Bari Musur* 4). To achieve the goals, first the transformation of lentil was performed in order to test the functional integrity of the old pSCP1 construct containing *Ri-pgip* and *bar* genes. Then transformation of lentil with the marker free construct was carried out in order to develop a selectable marker free transformation system. Transgenic plants were subjected to various molecular and functional characterizations to study and prove stable introduction and inheritance of the gene of interest to the following generations.

4. MATERIALS AND METHODS

4.1 Chemicals and Apparatus

4.1.1 Growth media

Compound	Company
MS basal salts mixture	DUCHEFA
B5 vitamins	DUCHEFA
Plant agar	DUCHEFA
D(+) saccharose	Carl Roth

Table 7: Growth media.

4.1.2 Plant hormones and additives

Compound	Molecular weight	Company	Solvent
IBA	203.2	DUCHEFA	KOH
NAA	186.2	DUCHEFA	KOH
Kin	215.2	DUCHEFA	KOH
BAP	225.3	DUCHEFA	KOH
TDZ	220.2	DUCHEFA	KOH
GA3	346.4	DUCHEFA	KOH
Glufosinate-ammonium (PPT)	198.16	Riedel DeHaen	dd H_2O
BASTA® (200g/l)		Aventis GmbH	dd H_2O
L-Tyrosine	181.2	DUCHEFA	dd H_2O/ NaOH
IPTG (isopropyl-β-D-thiogalstopyranoside)	238.3	Applichem	
X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside)			dd H_2O

Table 8: Plant hormones and additives.

4.1.3 Antibiotics

Substance	Molecular weight	Company	Solvent
Ampicillin	371.39	DUCHEFA	dd H_2O
Combactam	582.6	Pfizer	dd H_2O
Kanamycin	428.4	DUCHEFA	dd H_2O
Streptomycin	1457.4	DUCHFA	ddH_2O
Ticarcillin		DUCHEFA	dd H_2O
Tetracycline	480.9	DUCHEFA	EtOH

Table 9: Antibiotics.

4.1.4 GUS –assay buffer

100 mM Sodium phosphate buffer (pH 7.0),

0.5 mM Potassium ferrocyanide,

10 mM EDTA

1 mM (0.5 mg/ml) X-*GLUC* (dissolved in DMSO before adding to Gus buffer)

4.1.5 Enzymes and buffers

4.1.5.1 Restriction enzymes

Enzyme	10x Buffer	Company
BamHI	10x Unique buffer	MBI Fermentas
EcoRI	10x Unique	MBI Fermentas
HindIII	10x R^+ (red)	MBI Fermentas
KpnI	10x Unique buffer	MBI Fermentas
NcoI	10xY (Yellow) buffer	MBI Fermentas
NheI	10xY (Yellow) buffer	MBI Fermentas
NotI	10x O^+ (orange)	MBI Fermantas
PstI	10x O^+ (orange)	MBI Fermantas
SacI	10x Unique buffer	MBI Fermantas
Shrimp alkaline phosphatase (SAP)	10x SAP buffer	MBI Fermantas
T4 DNA ligase	10x ligation buffer	MBI Fermantas
XbaI	10xY (Yellow) buffer	MBI Fermentas

Table 10: Restriction enzymes.

4.1.5.2 Polymerase Enzymes

Enzyme	10x Buffer	Company
Taq Polymerase (Combizyme)	10 x opti buffer	Invitrogen
Taq Polymerase (Red Taq)	10x BioTherm™	Natutec
Taq Polymerase (Go Taq)	5x Flexi buffer (Green)	Promega

Table 11: Polymerase Enzymes.

4.1.6 DNA markers

DNA marker	Concentration	Company
Gene Ruler™ 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler™ 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas
DNA MB grade fish sperm	10 mg/ml	Roche Diagnostics

Table 12: DNA Markers.

4.1.7 Solvents, sterilizers and other

Compound	Company
Dimethyl sulfoxide (DMSO)	SERVA
KOH	Carl Roth
NaOH	Carl Roth
NaOCl	Riedel de Haen
EtOH	Roth

Table 13: Solvents, sterilizers and other.

4.1.8 Primers

Base	Primer	Sequence	Product
bar-Gene from *Streptomyces Hygroscopicus*	*bar*447- f *bar*447- r	5'-GATTTCGGTGACGGGCAGGA-3' 5'-TGCGGCTCGGTACGGAAGTT-3'	447 bp
bar-Gene from *Streptomyces Hygroscopicus*	*bar*- f *bar*- r	5'-GCAGGAACCGCAGGAGTGGA-3' 5'-AGCCCGATGACAGCGACCAC-3'	260 bp
Lentil histone	Lens his 260 Lens his 680	5'-TCTCAGATGGTGAAGGACGC -3' 5'-CTACAGCTGCAGTCTTGGCA -3'	420bp
Kanamycin resistance	NptI-f NptI-r	5'-GAAAAACTCATCGAGCATCA-3' 5'-TTGTCCTTTTAACAGCGATC-3'	400 bp
pGreen plasmid	pGII 297-f pGII 303-r	5'-GTTGGGTAACGCCAGGG-3' 5'-GGAGCTCGCCTGCTGGTCACTGG-3'	~1300 bp[1] ~2294 bp[2]
pGEM plasmid	T7 SP6	5'-CGACTCACTATAGGGCG-3' 5'-CACTATAGAATACTCAAGC-3'	~200 bp[1] ~ 1200bp[2]
	M13 f M13 r	5'-GTAAAACGACGGCCAGT-3' 5'-GGAAACAGCTATGACCATG-3'	
PGIP Gene from *Rubus idaeus* L.	*r- PGIP* 1 *r- PGIP* 366	5'-ATGATGGACTTCAAGCTCTT-3' 5'-CTTGAGATGTTTAAGCTTGG-3'	365 bp
PGIP Gene from *Rubus idaeus* L.	pSCP1 108 pSCP1 733	5'-CAAGACAGCCTTCAACAACCC-3' 5'-CCACAATCTGGGTGGTCTTGT-3'	625 bp
PGIP Gene from *Rubus idaeus* L.	*r-PGIP*421 *r- PGIP* 958	5'-CAGCTCAAGAACCTCACATT-3' 5'-GGTTATGGAAATACGACGTG-3'	537 bp
PGIP Gene from *Rubus idaeus* L.	*r-PGIP* 1(25) *r- PGIP* 749 (25)	5'-TGATGGACTTCAAGCTCTTCTCCCT -3' 5'-CATGTTCCTCGACAGATCCACAATC -3'	748 bp
PGIP Gene from *Rubus idaeus* L.	*r-PGIP*154(25) *r- PGIP* 805 (25)	5'-ACGCCGACTGCTGTACCGACTGGTA -3' 5'-CAAGTCCACGGCTCTCAAGCTGGTC -3'	650 bp
pSCP1 plasmid	Pscp1*BamHI*f Pscp1 *BamHI* r	5'-AAGGGATCCATGATGGACTTCAAGCTCTTCTCCC-3' 5'-TATGGATCCTTACTTGCAACTTGGGAGGGGAGC-3'	998 bp
Agrobactrium tumefaciens	PIC A1 PIC A2	5'-ATGCGCATGAGGCTCGTCTTCGAG -3' 5'-GACGCAACGCATCCTCGATCAGCT -3'	600 bp
Kanamycin resistence gene *npt* III	Kan/bin999 Kan/bin 1266	5'-AAGATTATACCGAGGTATG-3' 5'-CATTAGTCCATGCAAGTTT-3'	267 bp

Table 14: Primers.

1 Plasmid without insert
2 Plasmid with insert

4.1.9 Apparatus

Apparatus	Manufacturing Company
Autoclave	Tuttnauer, Systec 5075 ELV
Balances	Sartorius
Centrifuge	Sigma 302K
Deep freezer –80 °C	Lozone
Dry oven	Memmert, Model 400
Electrophoresis chamber	Bio-RAD
Electrophoresis power supply	Bio-RAD
Gel Documentation	Intas
Hybridization oven	Biometra / H.Saur Laborbedarf
Ice machine	ZIEGRA
Incubator	Heidolph Incubator 1000
Lab centrifuge	Eppendorf 5415C
Magnetic stirrer	Heidolph
Microwave	Thomson
pH meter	HANNA
Pipette	Eppendorf, Gilson
Refrigerator 4 °C	LIEBHERR
Rinsed water station	MILLIPORE
Sonicator	SonoRex RK255S
Spectrophotometer	Pharmacia Biotech, Ultraspec 3000
Stereomicroscope	Leica Wild M3Z
Thermocycler PCR	Biometra®
Thermostat shaker	Heidolph Unimax 1010
UV-Transilluminator	Vilber Lourmat
Vacuum pump (~100 mbar)	ABM
Vortex	Heidolph
Water bath	GFL®
Glass bottles	Schott
Scalpel blade	AESCULAB® No.11
Stock solution vessel	NALGENE® CRYOWARE™
Sterilization filter	MILLEX®-GS 0.22µM
Substrate	Goldflora, Oldenburg
Filter paper	Schleicher & Schuell
Parafilm	NESCO film
Disposable plastic wares	Greiner, Kitzel, Sarstedt

Table 15: Apparatus.

4.2 Plasmid construction and cloning

4.2.1 Ingredients

Sterile Luria Broth (LB) media

SOC media

100 mM $CaCl_2$, at 4 °C

86 % and 10 % sterile glycerol

4.2.1.1 SOC Media	4.2.1.2 LB (Lauria Broth) (Sambrook *et al.* , 1989)	4.2.1.3 YEP (Yeast Extract Peptone)
20 g/l tryptone 5 g/l yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgSO_4$ x 7 H_2O 2.033 g/l $MgCl_2$ x 6 H_2O 20 mM glucose (filter sterilized, freshly added before using)	10 g/l tryptone 5 g/l yeast extract 8 g/l NaCl pH 7.2	10 g/l tryptone 10 g/l yeast extract 5 g/l NaCl pH 7.2

Table 16: Mediums for bacterial cultures.

LB and YEP media were solidified by addition of 15 g/l Agar agar to prepare solid media.

4.2.2 Competent *E. coli* cells preparation for transformation

To prepare competent cells protocols of Nakata *et al.*, 1997 and Tang *et al.*, 1994 was followed.

1. The required *E. coli* strains (Top10) was grown overnight in 5 ml of LB medium with streptomycin 50 mg/l at 37°C to stationary phase.
2. The overnight culture was diluted in fresh LB 1:50 and grown at 37 °C until $O.D_{600}$ reached to 0.3 - 0.4.
3. The cells were harvested by centrifugation for 10 min at 4°C, 5600 rpm.
4. The supernatant was discarded and the pellet was re-suspended in 1/2 volume ice-cold

100mM CaCl2 and incubated on ice for 20 min, then centrifuged again.

5. Pellet cells were re-suspended in 1/10 volume ice cold 100mM $CaCl_2$ and incubated on ice for 1 hour and used immediately for heat shock transformation.

6. Alternatively, 86 % sterile glycerol was added to a final concentration of 15 % and then aliquots of 100 µl in 1.5 ml tubes, which were carefully placed in liquid nitrogen immediately to avoid loss of competency of the cells, afterward stored at -80 °C for long-term storage.

4.2.3 *E. coli* transformation - Heat shock/Calcium chloride method

1. Competent *E. coli* cells were taken from the -80 °C freezer and kept on ice to avoid melting.

2. 50ng (1-5 µl) of ligation mixture (or ready plasmids) were taken in a 1.5 ml Eppendorf tube and 50 µl of competent cells were added to it and was mixed gently.

3. The tube was incubated on ice for 20 min

4. Then placed in a water bath at 42°C for 30 seconds, extreme care was taken not to shake, returned immediately back onto ice for 2 minutes to avoid any cell damages.

5. 950 µl of pre-cooled SOC medium without antibiotics was added to develop antibiotic resistance and to reduce damage of *E. coli* cells.

6. Finally, the tubes were incubated on a shaker at 37 °C for 90 min at 250 rpm.

7. 100 - 200 µl of the resulting culture was spread on LB plates supplemented with appropriate antibiotic and was grown overnight at 37 °C. The colonies were ready to pick after 14 - 16 hours later.

4.2.4 *Agrobacterium tumefaciens* EHA105-pSoup competent cells preparation for electroporation

The hypervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.,* 1993) was co-transformed with the pSoup helper plasmid according to the pGreenII system (pGreen website, Hellens *et al.,* 2000).

1. An overnight seed culture was prepared by 2x10ml YEP supplemented with 5 mg/l tetracycline incubated with 250 µl of glycerol stock of EHA-105-pSoup at 28 °C on a shaker.

2. 2 ml of overnight culture was added to 50ml YEP with antibiotic and was grown for 3-5 h at 28°C to an $O.D._{600}$ of 0.4- 0.5.

3. Bacterial pellet was obtained by centrifugation at 4500 rpm at 4°C for 10 min, re-suspended twice in 25 ml ice-cold 10 % glycerol.

4. Centrifugation for 10 min at 4°C at 4500 rpm.

5. The pellet was then re-suspended twice in 2.5 ml ice-cold 10 % glycerol

6. Finally, the pellet was re-suspended in 1 ml ice-cold 10 % glycerol.

7. Aliquots of 100 µl were made in 2 ml Eppendorf tubes and transferred immediately into liquid nitrogen and stored at -80 °C for long term storage.

4.2.5 *Agrobacterium* transformation through electroporation

1. Competent *Agrobacterium* (EHA105-pSoup) cells were taken out from -80°C freezer and kept on ice to avoid melting.
2. 50 ng (1-5 µl) of plasmid solution was gently mixed with 50µl of competent cells in a 1.5 ml Eppendorf tube (or similar).
3. The mixture was transferred to a pre-cooled 0.2 cm glass cuvette and electroporated in a BioRad electroporator at: 25 µF capacitor, 200-400 Ω (ohm) resistance and 1.25 - 2.5 KV. The pulse field strength was between 6,25 – 12 kV/cm for 4-8 msec.

4. 500µl of pre-cooled SOC medium (without antibiotic) were added immediately and incubated on ice for 30 min.
5. Then the mixture was transferred to a new 2 ml tube and incubated for 3 hours at 28°C on a shaker at 250 rpm.
6. 100- 200µl of the resulting culture was spread on YEP plates (with the appropriate antibiotic-Kanamycin) and grown overnight at 28°C. The colonies were ready to pick after 24-48 hours.

4.2.6 Inoculation and harvest of *Agrobacterium*

25 ml YEP medium in 100ml Erlenmeyer flask including appropriate antibiotics for the respective plasmid (50 mg/l kanamycin for pSCP1-*Ri-pgip* and pGII0035S-*Ri-pgip*) was inoculated with 250µl glycerol stock of *Agrobacterium* containing the plasmid and placed on a shaker at 250 rpm in the dark for 19 h at 28°C. Bacteria were harvested by centrifugation at 4500 rpm. The supernatant was discarded and the pellet was re-dissolved in liquid B5-i medium (see 2.6.1.2) supplemented with 3.24 µM BAP or 5 µM TDZ. O.D600 was measured with a spectrophotometer and adjusted to 1-1.3.

4.2.7 Preparation of glycerol stocks of bacteria

Glycerol stocks of bacteria were prepared in a ratio of 1:3; a single colony was picked from the master plate, dissolved in 2 ml YEP or LB medium and was incubated for 2-3 hours on a shaker at 250 rpm. It was then transferred to 25 ml of the respective medium containing the necessary amounts of antibiotics and again incubated on a shaker at 250 rpm, 28 ºC or 37 °C for 19h in the dark. The stocks were prepared in 2 ml cryogenic vials (Cryoware-Nalgene, Rochester, USA) using 500µl sterile glycerol (86 %) and 1000 µl of growing bacterial-suspension and were stored at -80 °C for future use.

4.2.8 Maintenance of the plasmid and *Agrobacterium*

Since legume transformation is highly laborious and time consuming, it is advisable to check the correct insertion of the plasmid by restriction digest or sequencing and from time to time

preparing stocks from checked colonies. Plasmid isolation (4.3.8.2) was performed according to Birnboim and Doly (1979).

4.2.9 Binary vectors

In the following the T-DNAs of the plasmid used for the transformation are shown. Outside of the T-DNA of all represented plasmids, contains the NPT III gene. It originates from *Enterococcus faecalis* and encodes for an Amino-glycosid-3' - Phosphotransferase of type III, the one that is resistant against Kanamycin, Neomycin, Amikacin and other antibiotics of this class. All vectors used for the transformation are based on the vector pGPTV (Baker *et al.*, 1992), a derivate of the pBIN19.

4.2.9.1 pBI 121

Agrobacterium tumefaciens strain LBA 4404 contains plasmid pBI121 of 14KDa (binary vector) (Fig. 6) This binary vector contains within its right(RB) and left border(LB) the *uidA* gene (Jefferson *et al.,* 1986) encoding GUS (β –glucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene is used to asses the efficiency of transformation. A second gene *nptII* (Herrerra-Estrella *et al.*, 1983) encoding neomycin phosphotransferase II conferring Kanamycin resistance, driven by NOS terminator and promoter.
The bacteria also contain plasmid pAL4404 which is a disarmed Ti plasmid (132 KDa) containing virulence genes. The strain was provided by Prof. Zeba I. Siraj of Department of Biochemistry, Dhaka University, Dhaka, Bangladesh.

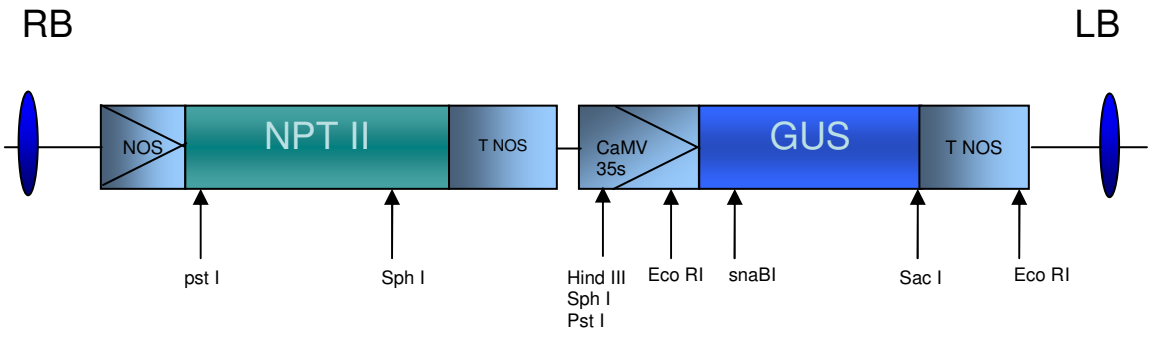


Fig 6: Schematic presentation of T-DNA of the plasmid pBI121.

4.2.9.2 pSCP1

This plasmid contains a *PGIP* gene from the raspberry (*Rubus idaeus* L.), on the T-DNA it codes for a polygalacturonase inhibitory protein (Williamson *et al.*, 1993; Ramanathan *et al.,* 1996). The gene is under the control of a double 35S promoter (Fig. 7). The genetic construct was made available within the scope of the EU project PRELEG by the Scottish Crop Research institutes (SCRI) (Richter, 2005).

In addition, the plasmid contains the *bar* gene (Thompson *et al.*, 1987) from *Streptomyces hygroscopicus* as a selective marker gene. The *bar* gene is under the control of a constitutive Nopalin-Synthase activator from *Agrobacterium tumefaciens*. The *bar* gene encodes for the Phosphinothricinacetyltransferase, it works by acetylation of Phosphinothricin(also Glufosinate). PPT binds as a structure analogue of the glutamate irreversible to the catalytic pit of the Glutaminsynthetase and acts in a way to the ammonium accumulation and leaf damages in the light (Hock *et al.,* 1995). Nevertheless, the crucial phytotoxic effect of PPT seems to be however depletion in glutamine by which the oxidative C_2-carbon cycle as well as the amino acid production is blocked. PPT is an active agent of the complete herbicides BASTA® and Liberty®.

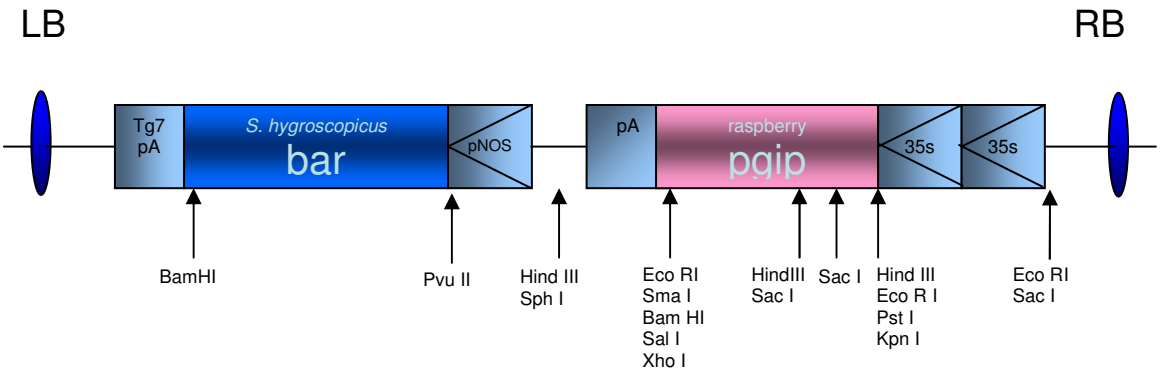


Fig 7: Schematic presentation of T-DNA of the plasmid pSCP1

4.2.9.3 pGreenII

pGreenII/ pSoup was also used in the present study. This is a dual-binary vector system (Hellens *et al.*, 2000). pGreenII has advantage over the other vectors due to its smaller size, easier handling, multiple cloning sites, high copy number and improved stability in *E. coli*. Under non-selective condition the number of *Agrobacterium* colonies containing a pGreen plasmid is reduced by 50 % after 24h which enhances the safety used of this vector (Hellens *et al.*, 2000). Since pGreen system is dual and needs presence of pSoup in the same strain which is providing replication functions *in trans* for pGreen.

The system gives another advantage of using pSoup for co-transformation to produce marker-free transgenic plants by a second T-DNA containing the marker while the gene of interest in pGreen (Vain *et al.,* 2003; Afolabi *et al.,* 2005). The prerequisite for this technique is a high efficient transformation protocol which serves high numbers of different transgene localization of the two T-DNAs.

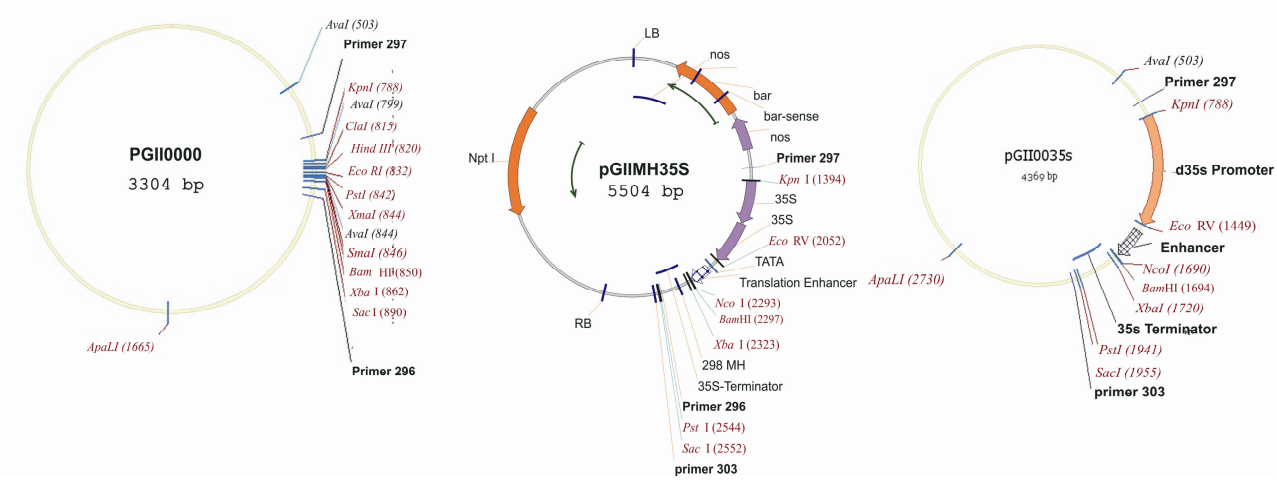


Fig. 8: Functional maps of the pGII 0000, pGIIMH35s and pGII0035s vectors used in the cloning work.

The T-DNA of pGII MH35s contains the *bar* gene fused between the *nos* promoter and terminator sequences of *Agrobacterium tumefaciens*. The *bar* gene encodes a phosphinothricin acetyltransferase (PAT) enzyme which confers resistance to bialaphos and the related compounds phosphinothricin (PPT), the active ingredient of the herbicide BASTA® and glufosinate ammonium through acetylation (Fig. 8). This part of the T-DNA cassette was removed and double 35s promoter and terminator region was taken and cloned into pGII, the total empty pGII vector to prepare pGII0035s.

4.2.9.4 pGEM

Naturally occurring (unmodified) plasmids often lack several important features that are required for a high quality cloning. These features are (1) a small size, (2) unique or single restriction endonuclease recognition site and (3) one or more selectable genetic markers. Nowadays, a number of artificially constructed plasmids are used as cloning vectors. All of these

plasmids contain one or more antibiotic resistance genes. Commonly used antibiotics for selection are tetracycline, ampicillin, chloramphenicol, kanamycin or neomycin.

pGEM-T Easy is used as a helping vectors (Fig. 9). It is available from the company (Promega Corporation, USA). The T overhang of this vector makes it easier for the PCR amplified clone product to be ligated. Moreover, the presence of multiple cloning sites within the Lac Z gene makes this vector efficient for blue white screening. This extra step in cloning of the GOI in pGEM and then use it to the desired vector is faster as the selection of the transformed clones (with GOI) is faster with pGEM.

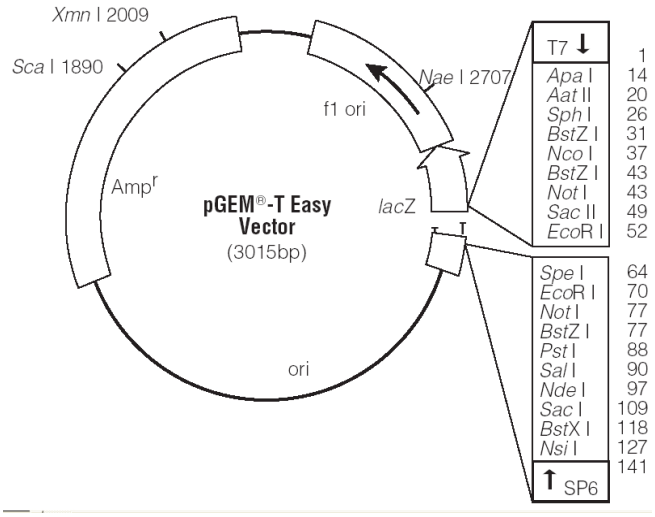


Fig. 9: Functional map of the pGEM-T easy.

The *Ri-pgip* gene was cloned into the Ti-plasmid using PCR; the *Ri-pgip* gene was amplified using two cloning primers pSCP1*BamHI* forward:

5'-AAGGGATCCATGATGGACTTCAAGCTCTTCTCCC-3' and pSCP1 *BamHI* reverse: 5'-TATGGATCCTTACTTGCAACTTGGGAGGGGAGC-3' flanking *BamHI* restriction site (underlined) to the PCR product using proof reading Combi*Zyme* DNA polymerase (Invitek GmbH, Germany). The protocol from the manufacturer was followed to prepare the PCR mixture.

4.2.9.5 PCR reaction mixture

Compound and concentration	Amount per reaction
Double distilled water	28.0 µl
10x PCR buffer	5.0 µl
50 mM $MgCl_2$	2.5 µl
10 mM nucleotides mixture (dNTPs)	1.0 µl
5X OptiZyme Enhancer	10.0µl
10 pmole forward primer	1.0 µl
10 pmole reverse primer	1.0 µl
20-50 ng plasmid DNA	1.0 µl
Combi*Zyme* DNA polymerase (4 U/µl)	0.5 µl
Total volume	50.0 µl

Table 17: PCR reaction mixture for insert (pDNA).

4.2.9.6 PCR program

Steps	Temperature (°C)	Time (s)	No of cycles
Initial denaturation	94	180	1
Denaturation	94	60	29x
Annealing	65 (primer specific)	60	
Elongation	72	60	
Final elongation	72	300	1
Cooling down after completion of PCR	4	∞	

Table 18: PCR program to amplify insert for cloning (pDNA).

The template for the PCR was pSCP1 containing the *Ri-pgip* gene. It was fused to a constitutive double 35S promoter of cauliflower mosaic virus (Wiese *et al.*, 1994). The PCR product was

purified directly using GFX™ PCR DNA and the Gel Band purification kit from Amersham Biosciences (UK) or from gel using the same kit.

4.2.9.7 Annealing temperatures for PCR

Primer	Annealing temperature (° C)
pSCP1 108 / pSCP1 733	53
r - PGIP 1 / *r- PGIP* 366	58
r - PGIP 1(25) / *r - PGIP* 749 (25)	60
r - PGIP 154 (25) / *r - PGIP* 804 (25)	60
r - PGIP 421 / *r - PGIP* 958	58
bar sense / *bar* antisense	62
bar 447 f / *bar* 447 r	62
Lens his 260 / Lens his 680	55
Kan/bin 999 / Kan/bin 1266	60
PIC A 1/ PIC A2	60
SP6/ T7	55
M13 f/ M13 r	60

Table 19: Annealing temperatures for PCR.

4.3 Molecular biological methods

4.3.1 Gel electrophoresis

4.3.1.1 TAE buffer (50x)	4.3.1.2 6x loading buffer (MBI Fermentas)
40 mM TrisHCl	50 mM EDTA
20 mM Glacial acetic acid	0.25 (w/v) % bromophenol blue
1 mM EDTA	0.25 % (w/v) xylene cyanol FF
pH 7.5	25 % (w/v) Ficoll40 (type 400, Pharmacia)
4.3.1.3 Ethidium bromide EtBr (stock 10 mg/ml, Roth)	

Table 20: Gel electrophoresis Buffers.

Electrophoresis is used to separate molecules (DNA and RNA) based on their size. Nucleic acids are negatively charged (anions) i.e. they will move towards the anode if current is applied. In agarose gel electrophoresis, the DNA is forced to move through a sieve of molecular pores made by agarose. The mobility will depend on the size and secondary structure of the DNA.
As DNA itself does not fluorescence, ethidium-bromide is added. This substance moves towards the cathode so it interacts and binds to double stranded nucleic acids. RNAs are smaller in size and are also negatively charged; they also bind with EtBr and are visualised as a smear (for their conformation as they are single stranded and becomes coiled while binding with EtBr) in the gel too. If analysed under UV light, DNA can be visualized as fluorescent band. The fluorescence increases with the amount of the DNA.

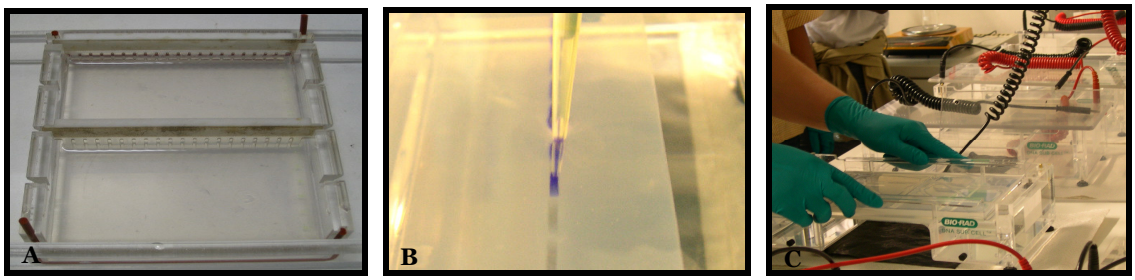


Fig. 10: Different steps of agarose gel electrophoresis: (A) Casting of gel (B) Loading samples in the wells (C) Gels in the electrophoresis tanks for running. Source: Author

4.3.1.4 Procedure for Gel electrophoresis:

1. An agarose gel with 1 X TAE 0.8-1 % (w/v) agarose was prepared, melted in a microwave oven until the agarose was completely dissolved.

2. The solution was cooled down until it reached a temperature about 60 °C then ethidium bromide (0.5 µg/ml final concentration) was added and the solution was casted into a gel casting tray to solidify.

3. A suitable comb was positioned to make slots (Fig. 10A).

4. After solidifying, the gel was transferred to the electrophoresis chamber containing running buffer (1x TAE buffer), enough to cover the gel completely. The comb was removed carefully.

5. Samples were prepared with 6x loading buffer and were loaded together with molecular

weight marker into the slots (Fig. 10B).

6. A voltage of 60-100 V was applied for 30-40 min for Electrophoresis and the run was stopped when the run had covered 2/3 distance of the gel (Fig. 10C).

7. Then the gel was taken out to observe the DNA fragments and documentation under the UV bench.

4.3.2 Digestion of DNA by restriction endonucleases

For molecular cloning, both the source DNA that contains the target sequence and the cloning vector must be consistently cut into discrete and reproducible fragments. It was only after the discovery of bacterial enzymes that cut DNA molecules internally at specific base pair sequences were discovered that molecular cloning became feasible.
DNA was digested using different restriction endonucleases with respective buffers as recommended by the supplier. When two enzymes had to be used for digest, the buffer was selected to be suitable for both enzymes; otherwise it was done one after the other. Digestion was done at 37 °C for 2 h or overnight, and then enzymes were heat-inactivated for 15-20 min at 65 or 85 °C, depending on the enzyme.

Ingredients	Amount per reaction
DNA	3 .0 µl
BamHI (10U/µl)	1.0 µl
HindIII (10U/µl)	1.0 µl
Buffer (10X)	1.0 µl
D.H2O	4.0 µl
Total	**10 µl**

Table 21: Digestion of plasmid DNA by restriction endonucleases

4.3.3 Purification of PCR product and DNA fragments

4.3.3.1 Purification of PCR product (Amersham)

500 µl capture buffer was taken in a GFX column in a collection tube. PCR product was added in it and mixed thoroughly by pipetting up and down, then centrifuged for 30 sec. at 12,000 rpm. The flow through was discarded. The spin filter was washed with 500 µl washing buffer by centrifuging for 30 sec. The collection tube was replaced with a 1.5ml Eppendorf tube. 50µl of elution buffer or TE buffer, pH8.0 was added directly on the glass matrix in the GFX column and incubated for 1 min at RT. Finally, centrifugation for 1 min at full speed to get a purified product.

4.3.3.2 Purification from agarose gel band

Agarose gel band slice weighed in a 1.5 or 2.0 ml tube. To the gel slice 10µl of capture buffer for each 10mg of gel slice was added and vortexed vigorously. This tube was incubated at 60°C in a water-bath for 5-15 min until agarose was completely dissolved. To melt the agarose, the tube was occasionally shaking thoroughly. When the agarose was dissolved completely, it was centrifuged briefly to collect the samples at the bottom of the tube. The agarose was transferred into a GFX column in a collection tube and was incubated for 1 min at room temperature. Then it was centrifuged at 12,000 rpm for 30 seconds in a lab centrifuge. Flow through was discarded. 500µl was added to the column and centrifuged again for 30 sec. The collection tube was discarded and replaced with a 1.5ml Eppendorf tube. 50µl of elution buffer or TE buffer, pH8.0 was added directly to the glass matrix in the GFX column and incubated for 1 min at RT. Finally centrifugation was done for 1 min at full speed to get purified DNA fragments.

4.3.4 Dephosphorylation of 5'-ends of digested vector DNA

To prevent re-ligation of the vector with the excised fragment as adapter shrimp alkaline phosphatase (SAP) was used to dephosphorylate of the 5'-ends of the digested vector. Dephosphorylation was done according to the manufacturer's protocol at 37 °C for 1h, and then the enzyme was heat-inactivated at 65 °C for 15 min.

4.3.5 Ligation

During the ligation reaction, fragments of foreign DNA carrying identical termini (either blunt-ended or with overhang) must be cloned in a linearized plasmid vector bearing compatible ends. The variety of restriction sites in plasmid vectors is now extremely large, and it is often possible to find a vector that carries exactly the same restriction sites as the fragment of foreign DNA itself. This has the advantage of allowing the foreign DNA to be recovered from the recombinant plasmid by digestion with the appropriate restriction enzymes. DNA ligases catalyze the phosphodiester bonds between a free 5'-phosphate group and a free 3'-hydroxyl group of the same strands of a dsDNA. Intramolecular ligation results in a circularization of the DNA molecule. If an insertion is planned, self-circularization and oligomerization has to be prevented by dephosphorylation or eluting the fragment from the gel.
Ligation of cohesive ends and the vector was done at a molar ratio of 3:1 in 5x ligation buffer, so 150 ng insert and 50 ng vector were mixed and 2U of T4 DNA ligase were added. The reaction was incubated at 22 °C overnight, and then the ligase was heat-inactivated at 65 °C for 15 min. A portion of the ligation product was monitored afterwards by running on a gel to check the efficiency of ligation and then was used for *E. coli* transformation (2.2.3).

Ingredients	Amount per reaction
Ligase buffer 10X	1.0 µl
T4 DNA ligase	1.0 µl
Insert	3.0 µl
Vector	1.0 µl
d.H_2O	4.0 µl
Total	10.0 µl

Table 22: Ligation mixture.

4.3.6 Selection of transformed colonies

On the next day of transformation the LB plates contained a mixture of blue and white colonies (only when pGEM vector was used). This tells the ligation was successful. After the recombinant plasmid vector was introduced in the bacteria, transformants need to be identified. For that purpose selection mediums are used. On a medium with antibiotic substance (Ampicillin), only

bacteria with plasmid(s) will form colonies, because the plasmid contains genes for antibiotic resistance. Since usually not all plasmids are recombinant, further selection is necessary i.e. selection of transformed bacteria for amplification (Ferl and Paul, 2000). For that purpose, the fact that plasmids also contain a functional *lac*Z gene is used. *Lac*Z gene codes for ß-galactosidase, an enzyme, which can hydrolyse a synthetic substrate X-Gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside) and as a result there is a blue coloured product.
Insertion of a gene fragment into *lac*Z gene inactivates the *lac*Z gene, and therefore bacteria containing recombinant plasmids will not be able to hydrolyse X-gal and the colonies will remain white (blue-white screening). Besides X-gal, IPTG (isopropyl-beta-D-thiogalactopyranoside) is also added to the selection medium, because it induces activity of ß-galactosidase, by binding to and inhibiting the *lac*Z repressor.

4.3.7 DNA preparation

4.3.7.1 Isolation of genomic DNA from plant tissue by the CTAB- based extraction method

Plant genomic DNA isolation is one of the basic requirements for the characterization of transgenic plants. The purity and the concentration of isolated DNA are important factors for the detection of the transgene. Total genomic DNA was isolated according to the CTAB method (Doyle and Doyle, 1990). For PCR screening, small scale (100-200 mg leaf material) DNA isolation was performed in this connection.

4.3.7.1.1 Buffers and Solutions

CTAB buffer	7.5 M NH_4 Acetate	0.5 M EDTA (pH 8.0)
3 % CTAB (added after autoclaving and stirred overnight) 1.4 M NaCl 0.2 % *β*-Mercaptoethanol (added directly before using) 20 mM EDTA 100 mM Tris-HCl pH 8.0 (base) 0.5 % PVP-40 polyvinyl pyrolidone (soluble)		

Table 23: Buffers and solutions for isolation of genomic DNA.

24: 1 CI Mix	Wash Buffer	RNAse A	TE Buffer + RNAse A
23 ml Chloroform 1 ml Isoamylalcohol	76 % Ethanol$_{Abs.}$ 10 mM Ammonium- acetate	10 µg/µl Stock sol. in ddH_2O	10 mM Tris-HCl, pH 8.0 1 mM EDTA 10 µg/ml RNAse A

4.3.7.1.2 Small scale genomic DNA (gDNA) isolation

1. Leaf material 100-200 mg was harvested in liquid nitrogen (either already frozen or fresh from greenhouse). The leaves were macerated to powder using pre-cold mortar and pistils and were transferred to 2 ml reaction tubes.

2. Under a fume hood 800 µl of preheated (60°C) CTAB-buffer were added to the samples and vortexed vigorously. Afterwards tubes were incubated for 30 min at 60 °C in water-bath.

3. 800 µl CI-Mix were added and tubes were gently mixed to avoid shearing of genomic DNA by inverting the tube for 4-5 times.

4. Centrifugation was done at room temperature for 10 min at 12,000 rpm and the aqueous phases (800 µl) were transferred into a fresh 1.5ml tube.

5. 2/3 volume (550 µl) of pre-cooled (-20 °C) isopropanol were added and gently mixed to allow precipitation of gDNA.

6. Centrifugation for 10 min (full-speed) at RT for pelleting gDNA.

7. The supernatant was discarded and the pellet was washed in 200 µl WB until the pellet swims.

8. The washing-buffer was carefully removed and the pellet was re-suspended in 200 µl TE buffer supplemented with RNAse A and incubated was for 30 min at 37°C.

9. 100 µl 7.5 M NH_4-acetate and 750µl of cold $EtOH_{abs.}$ were added and gently mixed to re-pellet gDNA. The samples were centrifuged again at full-speed for 10 min at room-temperature.

10. Supernatant was completely removed and the pellet dried for 40-50 min at 37°C.
11. Pellet was re-suspended in 100-200µl dd H_2O or 100 µl TE buffer (for better solving and storing) and kept overnight at 4°C dissolving completely.

4.3.8 Mini-preparation of plasmid DNA (modified after Birnboim and Doly 1979)

4.3.8.1 Buffers and Solutions

Solu A	Solu B	Solu C	Solu D
15 mM Tris-HCl pH 8.0 10 mM EDTA 50 mM Glucose 2 mg/ml fresh lysozyme	0.2 M NaOH 1 % SDS	3 M NaOAc, pH 4.8	0.1 M NaOAc, pH 7.0 0.05 M Tris-HCl pH 8.0 10 µg/ml RNAse A

Table 24: Solutions for plasmid isolation.

4.3.8.2 Procedure for Plasmid Isolation

1. 2ml of bacteria suspension was centrifuged at 12,000 rpm for 5 min and the supernatant was quantitatively removed.
2. The step was repeated using 1ml of bacteria suspension in the same tube.
3. The pellet was carefully re-suspended in 200µl of sol. A, and incubated for 15 min at RT.
4. Then 400µl of sol. B and 300µl of sol. C were added and mixed gently, followed by incubation on ice for 15 min.
5. The mixture was centrifuged twice for 10 min and the clear supernatant (800µl) was transferred into a new 1.5 ml Eppendorf-cap's and after spinning down for another 10 min supernatant was collected in a fresh tube.
6. 600µl cold isopropanol (-20 °C) was added to the supernatant and gently mixed till the DNA started precipitating.

7. To pellet Plasmid DNA centrifugation was done for 10 min and the supernatant was quantitatively discarded.
8. The DNA pellet was re-dissolved in 200µl of sol. D, and incubated for 5 min at RT.
9. 400µl $EtOH_{abs.}$ was added, mixed, and then was centrifuged for 10 min.
10. The pellet was washed in 200µl 70 % EtOH, then centrifuged again for 10 min.
11. The pellet was dried for 30-60 min at RT.
12. The pellet (plasmid DNA) was dissolved in 20-50µl of sterile deionised H_2O + 1µl RNaseA (1 mg/ml) or 50µl TE buffer + 1µl RNaseA and, the DNA quantity (10-20µg for *E. coli*) was estimated.

4.3.8.3 DNA quality measurement

The DNA measurement using a spectrophotometer is based on the fact that OD at 260 nm is twice than that of 280 nm if the solution contains pure DNA. The absorbance (*A*) of the DNA preparations was determined at 260 nm and 280 nm where *A260* = 1 is equivalent to about 50µg / ml for double-stranded DNA. The basic formula to measure DNA concentration is:

dsDNA-Concentration = (OD260 x Df x 50) µg/ml [here, Df = dilution factor]

The quotient *A260* / *A280* gives the level of DNA purity. The OD ratio between 260 and 280 nm decreases if there is any contamination from protein. Pure DNA has an OD260/OD280 between 1.8 and 2.0. A quotient below 1.8 indicates a contamination.
To check the suitability of isolated genomic lentil DNA for PCR, a single-copy gene encoding lentil histone protein primers (*Lens* his 260/*Lens* his 680, table 14) were used to amplify a 420bp fragment of HMG gene.
In addition to spectrophotometer, DNA concentration was also estimated in agarose gels. Sample DNA was applied and in parallel with fish sperm DNA dilutions (stock 10 mg/ml) was also applied in order to enable an estimation of the DNA-quantity in the gel.

4.3.8.4 PCR, colony PCR

PCR is used since late 80s. Kary Mullis with his colleagues working in Cetus Corporation USA invented PCR (Mullis *et al.*, 1986; Chawla, 2002). PCR is a relatively simple process by which virtually unlimited copies of selected DNA fragments using known sequence fragment (primers) can be generated and amplified *in vitro* in a short period.

Primers are short oligonucleotides (typically 18-22 bases in length) that are necessary to start the extension reaction in a specific manner. The reaction is carried out by a heat-stable *Taq*-DNA polymerase, named from *Thermus aquaticus*, the '*Taq*' thermophilic bacterium from which it was isolated and purified (Chien *et al.*, 1976). In PCR, poor yields and "mispriming" resulting in products which were often heterogeneous in size are quite frequently faced problem. These problems were solved with the introduction (Saiki *et al.,* 1988) of *Taq* polymerase.

4.3.8.4.1 PCR reaction mixture

Compound and concentration	Amount per reaction
Double distilled water	18.3 µl
10x PCR buffer with 50 mM $MgCl_2$	2.5 µl
10 mM nucleotides mixture (dNTPs)	1.0 µl
10 pmole forward primer	1.0 µl
10 pmole reverse primer	1.0 µl
20-50 ng template DNA (plasmid- or gDNA)	1.0 µl
1-2 U Taq DNA polymerase*	0.2 µl
Total volume	25.0 µl

* BioTherm Red Taq (10 U/µl) from Natutec.

Table 25: PCR reaction mixture (g DNA).

4.3.8.4.2 PCR program

Steps	Temperature (°C)	Time (s)	No of cycles
Initial denaturation	94	180	1
Denaturation	94	60	29x
Annealing	specific for GOI, primer	60	
Elongation	72	60	
Final elongation	72	300	1
Cooling down after PCR completion	4	∞	

Table 26: PCR program to amplify gDNA.

Colony PCR was used during cloning work as a rapid screening method for positive colonies. The same PCR reaction mixture was used and instead of DNA, a few cells from a single colony were picked using sterile pipette tip and mixed with PCR reaction mixture in PCR caps.

4.3.9 Functional Characterization of the transgenic plants

4.3.9.1 PGIP Assay

4.3.9.1.1 Buffers and solutions

Extraction Buffer	PG	Na-Acetate Buffer
25 mM Na-Acetate 1 M NaCl pH 5.0	Extracts from *Botrytis cinerea,* *Colletotrichum lupini,* *C. acutatum*	100 mM Na-acetate pH 4.6

Table 27: Buffers and Fungal Polygalacturonases for PG assay.

4.3.9.1.2 Protein Extraction

Fresh young leaf material from the greenhouse was harvested into liquid nitrogen and macerated in a pre-cooled mortar with a pestle (in liquid nitrogen). 1000µl of extraction buffer were added and vigorously vortexed immediately. Then the samples were incubated for 2 h at 4°C on a shaker. After incubation they are centrifuged for 10 min at 13,000 rpm to sediment the

coarse plant material. The supernatant was collected in a fresh tube and kept on ice. The extracted crude protein can be used directly for assay or stored at-20°C.

4.3.9.2 Measurement of the total protein with Bradford Assay

4.3.9.2.1 Equipment, reagent and solutions

Spectrophotometer (595 nm), plastic cuvettes, vortex, pipettes, falcon tubes (15 ml).

4.3.9.2.2 Reagents	4.3.9.2.3 Bradford stock solution	4.3.9.2.4 Bradford working solution
Coomassie Brilliant Blue G250 (Serva), Bovine serum albumin (BSA) stock 20 mg/ml (MBI Fermentas), 98 % ethanol 85 % phosphoric acid	100 mg Coomassie Brilliant Blue G250 50 ml 98 % ethanol 100 ml 85 % phosphoric acid	15 % (v/v) of stock solution in distilled water.

Table 28: Solutions for Bradford assay.

The total soluble protein concentration of the plant extracts was determined according to Bradford (1976). The maximum absorption of the dye (Coomassie brilliant blue G 250) changes from 465 to 595 nm in the presence of proteins in Coomassie blue's acidic environment. The reason for this is a complex binding between the dye and the protein. The Bradford-Assay is substantially more sensitive compared to the Lowry-or BCA (Bicinchoninic Acid)-Assay (Lowry *et al.*, 1951, Stoscheck, 1990). However, the disadvantage of the Bradford-assay is the fact that the same amounts in different standard proteins can lead to different absorption coefficients (Lottspeich and Zorbas, 1998). In the present work Bovine-serum-albumin (BSA, MBI Fermentas) was used as general standard, so all the samples had the same error and thus were comparable.

A standard curve was prepared using BSA at gradually increased concentrations of 0, 10, 20, 50, 75, 100, and 150 µg/ml in 100mM Na-acetate buffer. Protein samples were diluted 1:100 in 100 mM Na-acetate buffer, then 100 µl from the diluted samples were mixed with 4.9 ml working solution in 15ml falcon tubes, vortexed, and incubated. After approx. 10-minute incubation at

RT the absorption could be measured in the spectrophotometer. Absorbance was measured at 595nm and the standard curve was drawn and then the protein content was measured at A_{595} and calculated.

4.3.9.3 Absorbance at 280 nm (A280)

A rapid method was applied to determine presence of protein content in the samples (Wetlaufer, 1962). The correction for protein concentration can be done according to Schleif and Wensik (1981); the absorbance was measured at 280 nm and 260 nm when nucleic acid is present and then the protein content was calculated using the following formula:

Protein (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$

4.3.9.4 Agarose diffusion assay to prove the activity PGIP

The agarose diffusion assay to measure the activity of the polygalacturonase was

published by Taylor and Secor, 1988. The method was set up and optimized at the university of "La Sapienza" in Rome, in the working group of Mrs. Prof. Guilia De Lorenzo sets up and was further optimized in our lab (Richter, 2005).

4.3.9.4.1 Preparation of gel plates for the assay

For the assay a medium was prepared which was buffered in 100 mM Na-acetate (see 4.3.9.1.1) with a pH of 4.6. The medium contained 0.32% Agarose and 0.2% polygalacturonic acid from *Citrus* (Sigma P 3850). It was dissolved by heating in the microwave. After cooling, 45 ml of the media were poured out in a square Petri dish. After the medium was solidified, holes with a diameter of about 4-5 mm were punched with a cork borer.

4.3.9.4.2 Method of the assay

The assay was done by pipetting plant extract into the punched holes (Fig. 11). The plant extracts amount for a certain total protein amount (2000 ng -20,000 ng) were mixed with Na-acetate buffer and in each case the same amount (10µl) of fungal polygalacturonase were added

to it. As control, extract of a non-transgenic control plant with fungal polygalacturonase was used in the 2nd last hole on every plate and next place to it only the fungal polygalacturonase.

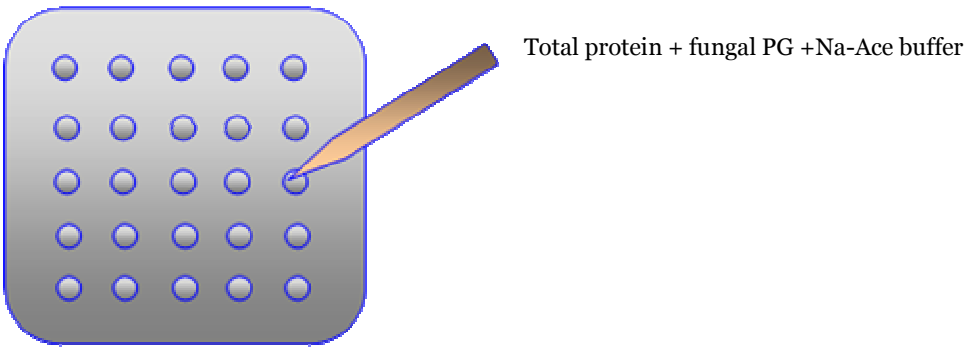


Fig. 11: The method of agarose gel diffusion assay.

Depending on the activity the used fungal Polygalakturonase, the plates were incubated for 18 to 48 h at 27°C.

4.3.9.4.3 Evaluation of the Agarose diffusion test – measurement of inhibition activity

At the end of the incubation period 6M HCl was poured on the plates and incubated for about 2 min. A halo becomes visible all around the holes for deactivation of the inhibition activity of the PGs, then the hydrochloric acid was removed and the results were evaluated. The halo around the holes showed the activity of fungal polygalacturonases (PG). The greater is the halo, the higher is the activity of the PG's. If the activity of the used PG's was inhibited by the addition of the plant extracts, this was to be recognized by a diminished halo size or with entire inhibition in the non-appearance of the halo. For evaluation, the diameters of the halos were measured with the help of a slide calliper and, were analyzed whenever appropriate, statistically.
The calculation of the inhibition on account of diminished halo size was done always in comparison to the halo size of the non-transgenic control. In the calculation the diameter of the punched out hole (4-5 mm) was deducted from the diameter of the halo.

Calculation of the inhibitory activity:

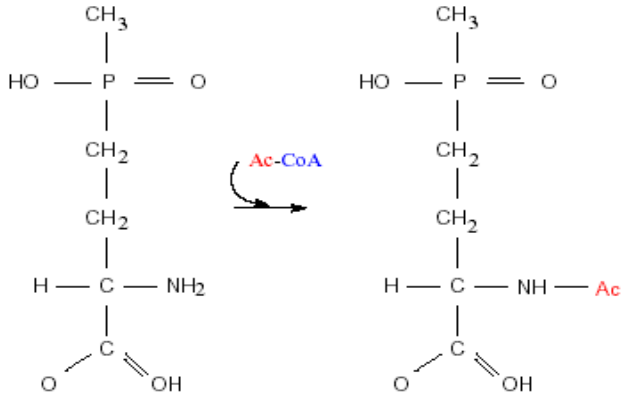
$$100 - \left(\frac{\text{Size of halo of transgenic plant (mm)}}{\text{Size of halo of control plant (mm)}} \right) \text{ x } 100 = \text{Inhibition Activity in \%}$$

4.3.9.5 Functional test for *bar* gene - Leaf paint assay

The pSCP1 construct used for transformation (Fig. 7) contains a *bar* gene as selectable marker gene. It encodes for the enzyme phosphinothricin acetyltransferase (PAT), isolated from *Streptomyces hygroscopicus*. It is analogous to the *pat* gene isolated from *S. viridochromogenes* (Murakami *et al*., 1986; Thompson *et al*., 1987; Strauch *et al*., 1988). Both enzymes confer resistance to bialaphos and the related compounds phosphinothricin (PPT), the active ingredient of herbicide BASTA®, Liberty® and glufosinate ammonium. BASTA® is a non-selective herbicide and has been regarded as environmentally safe (Nap and Metz, 1996). The *bar* gene offers an efficient and cheap selection system since all plants not containing or expressing *bar* will die.

Phosphinothricin inhibits Glutamine Synthetase (GS), the enzyme which incorporates NH3 into amino acids. When glutamine synthetase is blocked, the plants run out of amino acids and pH of the cell rises causing the plant/tissue death due to accumulation of NH_3.

Transgenic plants expressing *bar* gene are resistant to BASTA® as the enzyme covalently links an acetyl group to PPT to detoxify the compound (acetyl-PPT) (De Block *et al.*, 1987; Murakami *et al*., 1986) (Fig. 12).



PPT (MW: 181.13) acetyl-PPT (MW: 224.17)

Fig. 12: Detoxification and inactivation of PPT by acetylation. (Droege *et al.,* 1992).

The qualitative proof of the *bar* gene was carried out through coating the leaves with BASTA® (Aventis GmbH, Germany) at a dilution of 37.5, 75, 150, 300 and 600 mg/l (stock 200 g/l) with the help of a paintbrush, the opposite leaflet was marked as control (untreated) (Fig. 13). The evaluation was done after one week. The Leaf Paint assay was valued as negative if the leaf became wilted and showed the *bar* gene had no effect. The leaf paint counted as positive if in the

leaf no or only very low necrosis can be observed. With some plants, the evaluation of the Leaf-paints turned out difficult, because only the leaf edge or parts of the leaves were wilted, in these cases the result was noted as indifferent (+/-).

Fig 13: Possible results of the Leaf Paint tests. A leaf paint test was evaluated as positive if the leaf one week after application of BASTA solution was unscathed. If the leaf had wilted completely, the test pointed as negative. With partial necrosis the test result was classified as indifferent.

4.3.10 DNA sequencing and sequencing results

DNA (plasmid DNA and cDNA) was sequenced using different primers by MWG Biotech Company (Martinsried, Germany). The sequencing results were compared with the original sequence of *Ri -pgip* using Blast from NCBI website.

4.4 Bioinformatics and statistical programs

To analyse DNA-sequences, the freely accessible BLAST programme www.ncbi.nlm.nih.gov/BLAST was used. Restriction analysis of the DNA sequence was searched at http://tools.neb.com/NEBcutter2/index.php. Homolog-protein sequences from others organisms were also searched at www.expasy.org/BLAST .

4.5 Plant Material

4.5.1 Lentil seeds

The lentil (*Lens culinaris* Medik.) seed varieties of *Bari Musur* 1 (BM 1), *Bari Musur* 2 (BM 2), *Bari Musur* 3 (BM 3) and *Bari Musur* 4 (BM4) used in the present investigation were collected

from Bangladesh Agricultural Research Institute (BARI), Gazipur, Dhaka, Bangladesh.

When required shoot tip, epicotyl, hypocotyl, cotyledon, cotyledonary node, leaf, or embryo of the above materials were collected from aseptically grown seedlings.

4.5.1.1 Surface sterilization

The seeds were washed under running tap water for 3-5 min to reduce the level of surface organisms. Floating seeds were discarded; the remaining seeds were washed with distilled water. Lentil seeds were surface sterilized by soaking in 70 % ethanol (EtOH) (v/v) for 1 min followed by 6 % sodium hypochlorite (NaOCl) for 5-10 min, with agitation. Seeds were washed 5-6 times with sterile de-ionised water.

4.5.1.2 Seed germination

The surface sterilized seeds were required to be germinated to be used for transformation work.

4.5.1.2.1 Axenic culture

The surface sterilized seeds were cultured under sterile conditions on germinating media or on wet cotton or wet filter paper in Petri-dish and incubated in dark for overnight at ± 22°C.

4.5.1.2.2 Green house

The next generation seeds (T_0, T_1 etc) were germinated in pots containing garden substrates mixed with vermiculite (2:1) in the controlled environment of the green house.

4.5.1.3 Preparation of explants

Plantlets raised from seeds in axenic culture were the source of different kind of explants such as shoot tip, epicotyl, hypocotyl, cotyledon, cotyledonary node, leaf, embryo, decapitated embryo, longitudinal section (LS) of decapitated embryo etc. for regeneration and transformation experiments.

For all explant preparations seed coat was removed at the start and explants were prepared in the following way by excising with scalpel in aseptic condition from the growing seedlings:

1. Shoot tip - Shoot tips (appx. 2 mm) with few whorls of leaf premordia
2. Epicotyl – 5 mm long epicotyl (appx.)
3. Cotyledon – one segment of cotyledon
4. Cotyledonary node – cotyledonary nodes with cotyledon (appx. 3-4 mm)
5. Slited cotyledonary node – cotyledonary node with incision
6. Leaf – single young leaflet
7. Embryo- embryo from the matured seeds used for germination
8. Decapitated embryo – embryo with excised shoot tip and root tip
9. Embryo with single cotyledon disc – whole embryo with one cotyledon disc
10. Immature embryo - immature embryo from lentil plants grown in pots
11. LS of decapitated embryo with single cotyledon disc - A modified protocol of Schroeder *et al.,* (1993) and Bean *et al.,* (1997) was used for lentil transformation. Seeds were split open, shoot tips were decapitated and the remaining embryo axis was sliced longitudinally with a scalpel blade into two segments and the cotyledon disc attached to the embryo was kept (i.e. LS of decapitated embryo with single cotyledon disc). This was done to extend the area of cut surface.

4.5.1.4 Bacterial Inoculation and co -cultivation

The prepared explants were semi dried for about 45 - 60 min before adding bacterial suspension for inoculation.

Agrobacterium suspension re-suspended in B5i medium was poured into the Petri dish containing the dry explants. Incubation periods varied from 60-90 min. After that explants were blotted dry on sterile filter paper and transferred on Petri-plates with co- cultivation media for three days in the semi dark at 22±2 °C in a growth-room.

4.5.1.5 Washing

After co-cultivation, explants (white and white greenish colour) were washed several times (to remove surface bacteria) in sterile distilled water until the wash out water became clear. The final wash was supplemented with 300 mg/l Ticarcillin and incubated for 15 min on a shaker to

remove the still persistent *Agrobacteria*. Then the explants were blotted dry on sterile filter paper and cultured on MS regeneration medium supplemented with antibiotics for 3-5 days.

4.5.1.6 Introduction of selection pressure

After this regenerative phase the explants were subcultured on selection medium specific for the construct used.

The explants (LS of embryo decapitated at shoot end with single cotyledon disc), with healthy green sprouting were sub-cultured every two - three weeks to fresh medium with increasing concentrations of PPT to 2.5 mg/l, 5 mg/l and 7.5 mg/l. In brief the lentil transformation was done using the following scheme:

1. Explant preparation from mature embryos.
2. Partial dehydration of the explants
3. Inoculation with *Agrobacterium* suspension.
4. Co-culture 3 days / semi dark.
5. Washing and transfer onto fresh regeneration medium
6. First subculture 3-5 days/light/ medium with antibiotic to control bacterial overgrowth.
7. Second subculture 7-14 days/light / MS medium with antibiotic to control bacterial over growth.
7. Second subculture and first selection 14 -18 days/ light (MS).
8. Further subcultures to fresh media in three to four weeks

Selection

9. Transferred to Seramis or soil mixed with vermiculite.
10. T_1 seeds harvested after 30-45 days post transfer to pot.

4.5.1. 7 Selection agents

Kanamycin and Glufosinate-ammonium (PPT) were used as selective agents *in vitro* to select transgenic shoots.

4.6 Media

4.6.1 Media for Lentil transformation

4.6.1.1 Germination medium

MS macro- and micro salt's (Murashige and Skoog, 1962)
20 g /l sucrose
8 g/l Plant agar
pH 5.8

4.6.1.2 B5-i re-suspension medium

B5 basal micro- and macro salts (Gamborg *et al.*, 1968)
10 g/l glucose
10 g/l sucrose
2 g/l MES
pH was adjusted to 5.6 with 1N KOH/1N HCl

4.6.1.3 Co-cultivation medium

B5 basal micro- and macro salts
B5 vitamin mixture
30 g/l sucrose
pH was adjusted to 5.8 and the medium was solidified by adding 8 g/l Plant Agar.

4.6.1.4 MS regeneration medium

MS macro- and micro salt's (Murashige and Skoog, 1962)
B5 vitamin mixture
30 g/l sucrose
Different concentration (0.2 – 10 mg/l) and combinations of plant hormones were used eg. BAP, KN, GA_3, NAA, TDZ, Tyrosine for shoot induction and multiplication.
pH of the media was adjusted to 5.8 and the medium was solidified by adding 8 g/l Plant Agar. Post autoclaving and cooling to 60 °C, the medium was supplemented with 100 mg/l Ticarcillin and 100 mg/l Combactam.

4.6.1.5 Selection medium

MS basic micro- and macro salts
B5 vitamin mixture
30 g/l sucrose
pH was adjusted to 5.8 and the medium was solidified by adding 8 g/l Plant Agar. After autoclaving the medium was supplemented with 100 mg/l Ticarcillin, 100 mg/l Combactam and 2.5 mg/l PPT or 50 mg/l Kanamycin.

4.6.2 Root induction

4.6.2.1 Rooting medium

MS basic micro and macro salts full strength or half strength alone or with different concentration and combination plant hormonal supplementation were used for example IBA (0.98 – 122.5µM/l), IAA (0.57 – 114.2µM/l), NAA (0.54 –53.7µM/l) for root induction.
pH was adjusted to 5.8 and the medium was solidified by adding 7.5 g/l Plant Agar.
100 mg/l Ticarcillin, 100 mg/l Combactam, 5 mg/l PPT or 50 – 200mg Kanamycin were added when rooting medium used for transformed shoots.

4.6.2.2 IBA Shock Treatment

High concentration of IBA (980.0µM/l) was used to give a shock to the cut ends of the regenerated shoots for 10-20 min. Then the shoots were transferred to rooting medium with or without IBA.

4.6.2.3 Filter paper bridge

A Filter-paper bridge with a small hole was prepared on liquid rooting medium and *in vitro* regenerated shoots were placed through the hole so that the cut end of the shoot stay dipped in the medium.

4.6.2.4 Micro grafting

Stalk – about 1.54 cm tall stalk with root part from non –transformed germinated seedlings grown in dark.
Scion – Non-transformed or transformed single shoot.

Therefore, sterile lentil seeds of BM4 were germinated on water agar medium (0.4 % plant agar) in dark, the etiolated 5-7 days old plantlets were used as stalk for *in vitro* grafting. A vertical incision was made the middle of the stalk to allow the shoot to be grafted to fit. The cut end of the shoot was formed in 'V' with a sharp scalpel and carefully placed on the stalk. The join was tied up with a piece of sterile thread to keep the parts (scion and stalk) together in place. A drop sterile of IBA (122.5µM/l) was added to enhance the grafting process.

4.6.2.5 Transplantation

The plantlets having sufficient root systems or completely fixed grafts were taken out of culture vessels and washed in water to remove the attached agar from the roots. Then they were transplanted to small pots containing Seramis or substrate mixed with vermiculite (2:1) or garden soil mixed with sand and cowdung (1:2:1). The transplanted plantlets were kept covered with either *polythene* bags or plastic covers to prevent desiccation. To reduce sudden shock of environment change the plantlets were kept in the controlled environment of the growth room. Water was sprayed in every 24h to maintain the hyper humidity around the plantlets. Exposure to natural environment was done by removing the protective cover gradually after starting from 3rd day of transplantation. Finally, cover was removed completely after 7-10 days. The plantlets were developed to mature plants were the natural conditions of the greenhouse.

4.7 Nomenclature of the transformation experiments

In order to easily handle different transformation experiments and analyze different transgenic clones, a code or ID was used to differentiate between different clones and generations, the code used is: X -E (T_0), T_1, T_2, T_3 and so on, where:
X denotes the transformation experiment number
E denotes the T_0 plants

T_1 stands for the first transgenic generation

T_2 stands for the second transgenic generation

T_3 is for third generation and so on.

For example, the following code 14-35-5-3-1, 2, 3 is explained as follow: 14 is transformation experiment number, -35 is T_0 plant, -5 stands for the T_1 plant from seed number 5 of T_0 plant 35. The -3 denotes the T_2 from seed number of T_1 35-5 and -1 is the T_3 plant from seed number 1 of T_2 35-5-3. The number after the comma indicates different siblings of 35-5-3 generations.

5. RESULTS

5.1 Regeneration in Lentil

For any transformation work it is necessary to have a stable and efficient regeneration system of the plant material to be used. As legumes are of recalcitrant nature, it is important to have the initial regenerative system first.

5.1.1 Explant

A variety of explants were tried in the preliminary level of this investigation to find out one for regeneration suitable for transformation. Namely- cotyledonary node (CN), shoot tip (ST), epicotyl (Epi), slited cotyledonary node (SCN), embryo (Emb), embryo with single cotyledon disc (CE), decapitated embryo (DE) , immature embryo (IM), leaf (L), hypocotyl (Hyp) and cotyledon(C) were analyzed for their respective regeneration potential.

Immature embryos had to be removed from our work list as it was available only in the short growing season of lentil. Hypocotyl, epicotyl, leaf, cotyledon were also removed from the list as they did not show any response for shoot regeneration. The multiple shoot regeneration work was focused on with CN, DE, CE explants mainly. Final optimization of explant for transformation was made by modifying CE explant by slicing of the embryos longitudinally and decapitating the shoot tip leaving the root tip intact.

Four Bangladeshi lentil 'BARI Musur' varieties namely BM1, BM2, BM3 and BM4 were used for the present study in the beginning, but finally the work focused only on BM4. Other varieties were dropped because there were no significant differences among the 4 varieties during the regeneration experiments.

5.1.2 Multiple shoot regeneration in Lentil – Effects of plant hormones on multiple shoot regeneration in lentil

A total number of ~ 150 hormonal combinations were used in order to get multiple shoots in lentil, varying in concentrations from a range of 0.1 - 10 mg/l depending on the hormonal combination to be used. In most of the experiments 24 explants were subjected to inoculation for each hormonal combination.

Hormones used were - (A) Auxins: IBA, NAA, IAA; (B) Cytokinins: BAP, Kn, TDZ;

(C) Gibberellic Acid: GA_3; (D) Other additives: Tyrosine;

Figure 14 (pg.86) and 16 (pg.88) are showing the different responses towards multiple shoot regeneration from lentil cotyledonary node and decapitated embryo explants on media supplemented with different plant hormone combinations.

In MS media supplemented with BAP in concentrations from 0.88µM to 22.2µM/l, cotyledonary node (CN), shoot tip (ST), Embryo (Emb), Embryo with single cotyledon disc (CE) and decapitated embryo (DE) responded with low frequencies of shoot formation while leaf (L), epicotyl (Epi), hypocotyls (Hyp) and cotyledon(C) explants were forming succulent cells but no shoots. The number of shoot per explant varied from 5-6 for all the explants used, except cotyledonary nodes and decapitated embryos where the maximum number of shoot obtained was ±8 on 2.22 and 4.44µM/l BAP supplemented media. The shoot formation started about 7 days after inoculation on medium.

When BAP (0.44 – 22.2µM/l) was combined with NAA (0.54 – 2.27µM/l) the explants (CN, DE) responded by green mass shoot primordia and a few thin elongated shoots. In most of the cases there were callus like structures, which may have been shoot primordias, at the cut bases of the explants. The number of shoots varied from 4-6 per explant on media containing 4.44µM/l BAP and 0.54µM/l NAA. In this set of combinations again the CN and DE were responding better than E or ST explants. It was observed that increase in these hormones also increased formation of the callus like structure.

Since TDZ is known as miracle agent for plant regeneration and works effectively (Murthy *et al.,* 1998), this growth regulator was tried to analyze its effects on multiple shoot regeneration in lentil. A concentration range from 0.098 – 2.27 µM/l was used in MS medium for this purpose. Best response was obtained with media supplemented with TDZ 0.908µM/l. The results were green massive embryonic shoots from almost all type of explants, except hypocotyl, leaf, epicotyl and cotyledon explants. These cell clumps were compact in nature.

A combination of BAP (0.44µM – 22.2 µM/l) with TDZ (0.098 – 0.91 µM/l) was also used towards multiple shoot formation in lentil. ST, Emb, DE, and CN explants showed responses by forming numerous shoot primordia (≥50) but very few elongated shoots Epi, L, C and Hyp explants increased in volume but died after a few days. No shoot was formed from these explants. Best response was with 4.44µM/l BAP and 0.098 µM/l TDZ supplementation in the medium from CN explants. Shoot formation took place after an average of 7 days.

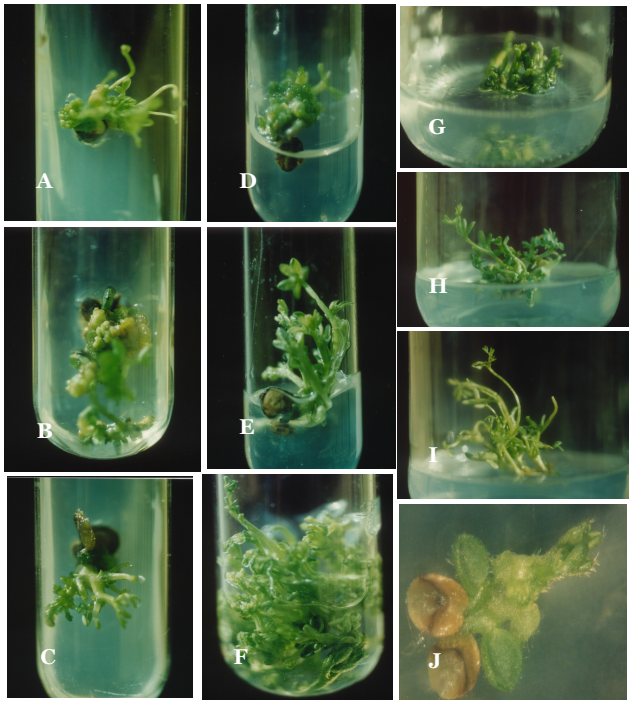


Fig. 14: Responses in multiple shoot induction in lentil cotyledonary node explants in the presence of growth hormones (concentration not shown)
(A) BAP
(B) BAP+NAA
(C) BAP+ Kn (D) BAP +TDZ
(E) BAP+GA_3
(F) BAP+ Kn +GA_3
(G) TDZ
(H) BAP+Kn+GA_3
(I) BAP+Kn+GA_3+ Tyrosine
(J) Regeneration from leaf explant

When Kinetin (0.93 – 4.65 µM/l) was used in combination with BAP, the responses among the explants were noticeable as good numbers of shoot were regenerated from CN, Emb, DE, CE and ST explants. 2.22 µM/l of BAP and 2.32µM/l Kn containing media gave rise to 6-8 initial shoots per explants.

BAP (0.88-4.44µM), Kn (0.93 – 4.65µM) and NAA (0.54 – 5.37µM) were also used in combination in search of response towards shoot regeneration in lentil. Variations in the numbers of shoot formed were observed according to the concentration of NAA. The explants were showing better responses by forming 4-6 shoots from CN, DE, E explants when MS medium contained 2.22µM BAP, 2.32 µM Kn and 1.07 µM NAA.

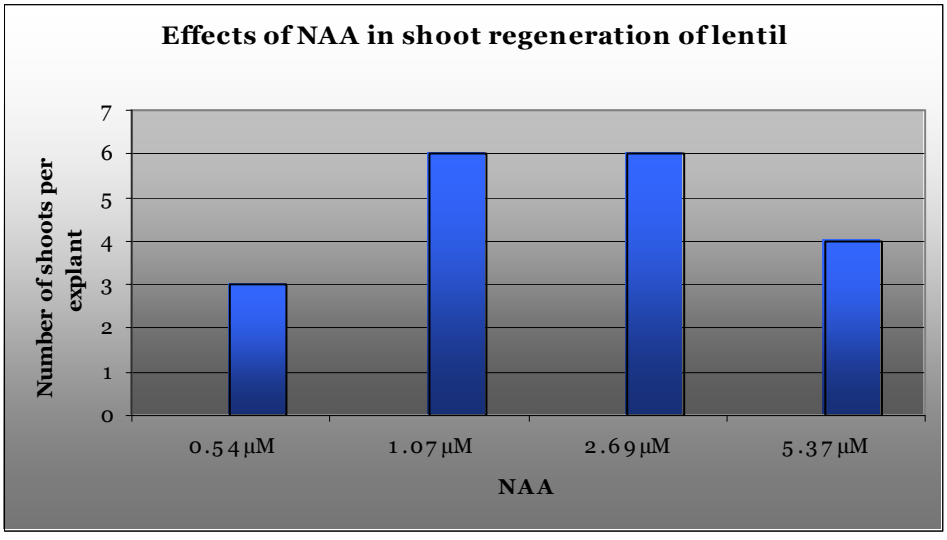


Fig. 15: Effects of NAA in shoot regeneration of lentil from cotyledonary node explant.

In figure 15, the varied response of the CN explant is shown. With the increase of NAA concentration the number of shoot reduced, while concentrations lower than 5.37µM were efficient in initiating shoots (statistics not analyzed).

Combination of BAP (0.88 – 4.44 µM/l), Kn (0.93 – 4.65 µM/l) and IAA (1.14 – 5.71 µM/l) were also tried on the CN, DE, E and ST explants for multiple shoot initiation. Again 2.22 µM/l BAP and 2.32 µM Kn together with 1.14 µM/l IAA were found working better towards shoot formation from the CN and DE explants. Shoots formed under these combinations showed good elongation and health.

Till now it was evident that BAP concentrations between 2.22 – 4.44 µM/l were more or less optimal. GA_3 (0.29 – 5.78 µM/l) was also used in combination with BAP. Shoot formation rates were 5-8 shoots per explant of cotyledonary nodes while ST, E and DE explants were forming 4-5 thin long shoots. Best response was obtained with 4.44µM/l BAP and 0.29µM/l GA_3. This showed that 0.29µM/l GA_3 was sufficient and helpful for shoot elongation during regeneration.

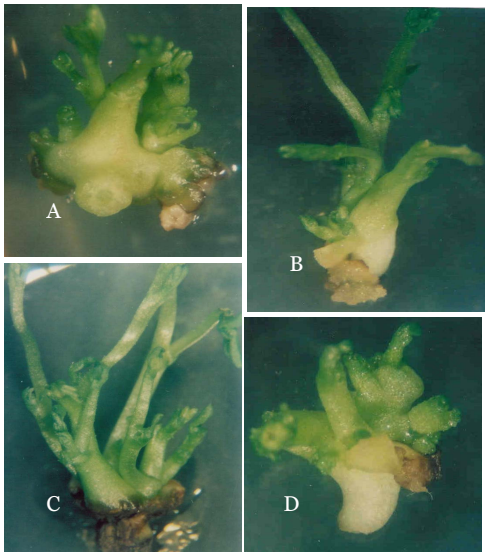


Fig. 16: Multiple shoot regeneration from decapitated embryos of lentil on
(A) BAP+Kn
(B) BAP+NAA
(C) BAP+Kn+GA_3+Tyr
(D) BAP+ Kn+IAA.

To further improve the efficiency of the media and to optimize the system the hormone combinations of 2.22 µM/l BAP and 2.32µM/l Kn were taken as base. GA_3 was added at 0.29 - 4.33 µM/l. The responses were checked against CN and DE explants as these two explants were found to be responding better than the other explants with respect to multiple shoot regeneration. With BAP, Kn and GA_3 the best result was achieved when BAP and Kn were combined with 0.29 µM/l GA_3. 6-8 healthy shoots with well expanded leaves were regenerated from the explants.

A further modification was done by adding tyrosine to the latest combination; this was done by adding 2.75µM/l – 30.25 µM/l tyrosine to the hormonal combination mentioned above. It was observed that from CN explants 10 -12 shoots were formed with 30.25 µM/l tyrosine supplementation. These shoots elongated sufficiently, leaf expansion was also better but the shoots had comparatively weaker stems than shoots on same medium without tyrosine. Addition of tyrosine increased the number of shoots per explant than shoots obtained in the previously used hormone combinations during the present work.

A summarized table (table 29) is given below for the responses of different lentil explants in presence of various plant hormonal combinations used in MS medium for multiple shoot regeneration.

Media	Hormone supplement	Variety	Explant	No. of shoots per explant	General Observation
MS	0.908 µM/l TDZ	BM2, BM4	CN , DE	Numerous	Embroid like clumps
	4.44 µM/l BAP + 0.098 µM/l TDZ	"	"	Numerous	As above with sudden 1 or 2 long shoots
	2.22 µM/l BAP + 2.32 µM/l Kn	"	"	6 –8	Healthy but dwarf shoots
	2.22 µM/l BAP + 2.32 µM/l Kn + 1.07 µM/l NAA	"	"	5 – 6	Dwarf shoots
	2.22 µM/l BAP + 2.32 µM/l Kn + 0.29 µM/l GA_3	"	CN, DE,CE	6 – 8	Comparatively longer shoots
	2.22 µM/l BAP + 2.32 µM/l Kn + 0.29 µM/l GA_3 + 30.25 µM/l Tyrosine	"	"	10 - 12	Thin long shoots

Table 29: Summarized table for multiple shoot regeneration in lentil.

To carry out the first transformation experiments, MS medium supplemented with BAP, Kn and GA_3 was selected to be used for co-culture as well as selection medium for the CN, DE and CE explants. But after modification of CE explant only MS medium was used for our further work. With intact root primordia plant growth can be obtained only on MS media while presence of any hormone had inhibitory effect on root growth.

5. 2 Rooting in Lentil

Rooting is complicated in case of lentil; moreover, no reproducible report has yet been published on lentil rooting so a number of experiments for root induction were initiated. Shoots that regenerated in various experiments during the study did not root spontaneously. To induce

root, individual shoot was excised and was cultured in various media containing different supplements of hormones.

5.2.1 Plant growth hormones and agar

IBA, IAA, NAA, GA3 were used either alone or in combinations in order to initiate root induction.

Normal treatments with different hormones at high concentration i.e., 122.5µM /l IBA was able to induce roots in *in vitro* raised shoots of lentil. Out of 36 explants 16 of them rooted, giving a success of 44.44% after 6-8 weeks. In the same time *in vitro* raised shoots from germinated seeds were showing better response by starting rooting normally in comparatively lower IBA concentration of 19.6µM/l (11.11%) but also with the high concentration 122.5 µM/l (50%). Table 30 is showing the varied response of the *in vitro* raised shoots of lentil in presence of different concentrations of IBA. A negligible rooting from the *in vitro* shoots of lentil was obtained with high concentration IBA (980µM) shock treatment. The formed roots were non-functional, all arising after initial callusing stage.

Besides IBA, other hormones were studied for rooting like NAA and IAA. From these two hormones NAA promoted root formation at concentrations of 2.69 – 8.06µM/l. A percentage of 6.66% was achieved from these concentrations. 8- 10 succulent roots were formed at the base of the shoots on media containing 8.06 µM/ l NAA. It was observed that the shoots were elongated and flowered on NAA containing medium though they showed no response to produce root. Seed set was observed, pods matured eventually. On the other hand IAA succeeded with 2.5% in root induction only with high concentration (114.2µM/l). The root type was similar to the one obtained with NAA but less in number, only 3-5 roots could be initiated. This concluded that IBA was comparatively efficient than NAA or IAA.

In vitro shoots were subjected to high concentrations IBA as a shock treatment (122.5 – 980µM/l). Subsequently the shoots were cultured on MS or ½ MS medium devoid of any hormonal supplement.

Media	Hormone supplement IBA (µM/l)	Explant		No of Explant	Days to root Initiation (in weeks)		No of rooted plant		Percentage	
					GS	INS	GS	INS	GS	INS
MS	0.98	Shoots from *in vitro* germinated seeds **(GS)**	*In vitro* raised shoots **(INS)**	36	X	X	0	0	0	0
	2.46			36	X	X	0	0	0	0
	3.67			36	X	X	0	0	0	0
	4.90			36	X	X	0	0	0	0
	9.8			36	X	X	0	0	0	0
	19.6			36	~8	X	4	0	11.11 %	0
	24.5			36	X	X	0	0	0	0
	49.0			36	~8	X	5	0	13.89 %	0
	73.5			36	~8	X	5	0	13.89 %	0
	98.0			36	~6-8	X	11	0	30.56	0
	122.5			36	~6-8	~6-8	18	16	50%	44.44%

Table 30: Responses of lentil shoots towards root formation in the presence of IBA.

5.2.2 Filter paper-bridge

Filter paper-bridges (Fig 17 F) were used over liquid MS medium containing 122.5µM/l IBA in order to create stress condition for the *in vitro* raised shoots as it is a common phenomenon in plants that they send their roots deeper under ground in search of water when there is scarcity of water. But rooting was not improve with this, most of the cases one single root was forming from the base after 6-8 weeks or more of incubation.

It was, however, interesting to find that the shoots were much healthier, better elongated with well expanded leaves. They branched when they were subjected to the rooting medium containing IBA. In addition to this these shoots showed *in vitro* flowering and ultimately they set seeds.

Another observation was that the initial media composition of *in vitro* shoots may also have an effect on root induction. For example, shoots grown on low concentration BAP, Kn, GA3 medium had better success than shoots from BAP or TDZ medium.

Except the grafting experiment (see 4.6.2.4) only MS + 122.5 µM/l IBA produced roots on the in-vitro regenerated shoots at a percentage of 44% while all other attempts failed or were of negligible frequencies together with non-functional roots, resulting in plant losses after transfer to soil.

It was also observed that there may be a seasonal effect on the root formation on the *in vitro* regenerated shoots. Rooting process was functioning only during the period between April to

July. It is possible that the controlled environment of the growth room cannot overcome this seasonal barrier, which may be due to unknown effects in the lentil life cycle.

5.2.3 Micro –grafting

As observed in the above results with different growth regulators it was decided to try micro-grafting of the *in vitro* raised shoots in order to avoid a rooting step and also to minimize losses during rooting as well as to recover whole plants in a relatively short time. For micro-grafting, 2-3 cm long stem/epicotyl with root part of seeds germinated *in vitro* were taken as 'Stalk' and the *in vitro* raised non transformed or transformed shoots were used as 'Scion'.

The micro-grafted explants were incubated on hormone free MS medium until graft setting (4-5 weeks) by growth of wound callus to close the cutting surface and were placed in the growth room. The grafted plantlets were transferred to pots containing soil and were covered with plastic bag to protect them from excessive water loss, and acclimatized gradually. The plants grew well and ultimately flowered and set seeds. The non transformed shoots gave a grafting result of 58.33% while it was only 16.67 % with the transformed shoots. Fig 17 is showing the attempts of the rooting experiments of the present study.

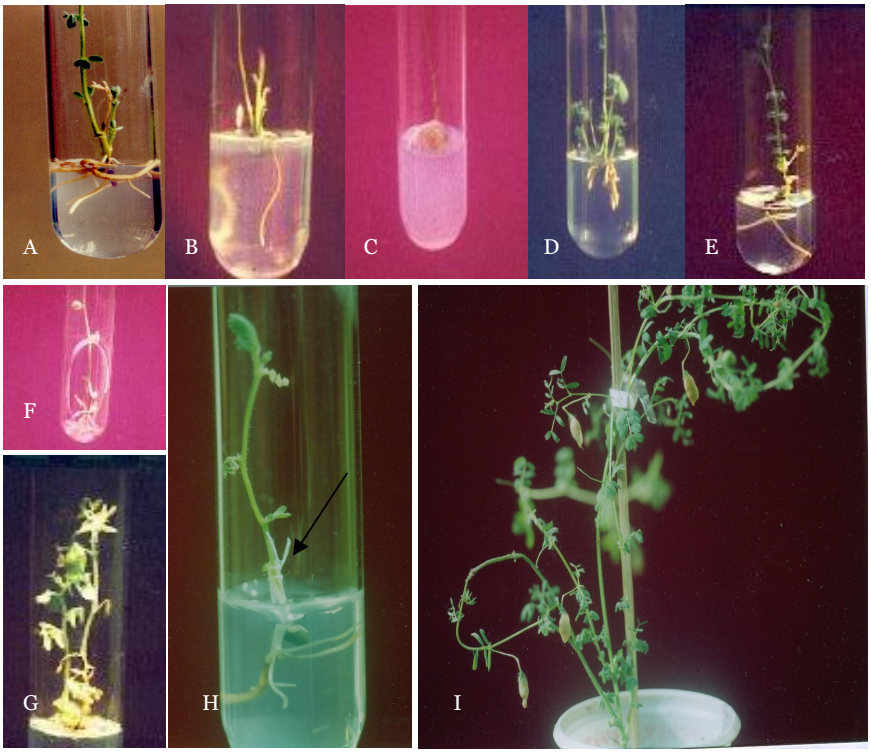


Fig. 17: Comparison of rooting attempts in lentil:

(A) Shoot from germinated seedling on 19.6µM/l IBA, *In vitro* regenerated shoot (B) on 114.2 µM/l IAA (C) on 19.96 µM /l NAA (D) on 122.5 µM/l IBA (E) on 19.6 µM IBA µM/l (F) Filter paper bridge and liquid MS with high conc^{n} IBA (G) *In vitro* seed setting on IBA rooting medium (H) Micro-grafting (I) Maturation of micro-grafted plantlet after transferred to soil.

As it is ultimately necessary to root the transformed shoots to make the purpose of transformation successful, a stable rooting system was absolutely necessary. From the above

mentioned results so far with the other experiments only micro-grafting had shown possibility to be used as a method, but this method is too time consuming and very tedious work and also not always can be done with perfection as lentil shoots are very thin and fragile making them difficult to graft. So, the whole regeneration system was changed by switching to 'Embryo with single cotyledon disc decapitated at shoot end' (Fig 18). Usually such plantlets were ready for soil transfer within 4 weeks. This also minimized the transfer time from *in-vitro* culture to pots for acclimatization.

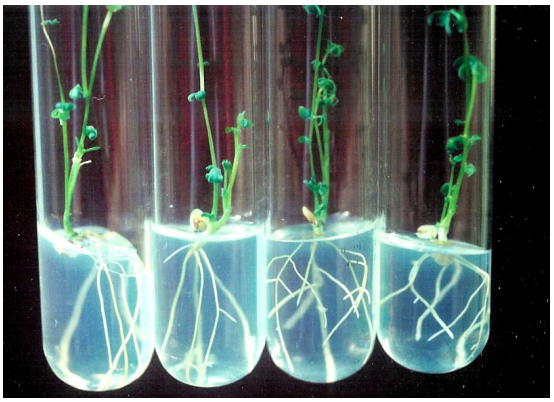


Fig. 18: Easy rooting of the LS of embryo decapitated at shoot end and with single cotyledon disc on hormone free MS medium.

This ultimately was the base of the further transformation work of the present investigation. This explant had advantage by growing roots normally for the intact hypocotyl of the embryo and a second advantage was only MS medium was required for growth.

5.3 Transient GUS expression after transformation with the construct pBI 121

Transient GUS assays were used to check the capability of BM lentil varieties for *Agrobacterium* mediated transformation. A total of 840 explants (cotyledonary node, decapitated embryo and embryo with single cotyledon disc) were co-cultured with *Agrobacterium tumefaciens* LBA4404 containing the β- glucuronidase gene (*gus*) compared with control explants. Based on the GUS assays, explants were found to show 60 -100 % transient GUS expression in explants transformed with GUS gene (Fig 19), while no activity was detected in control explants.

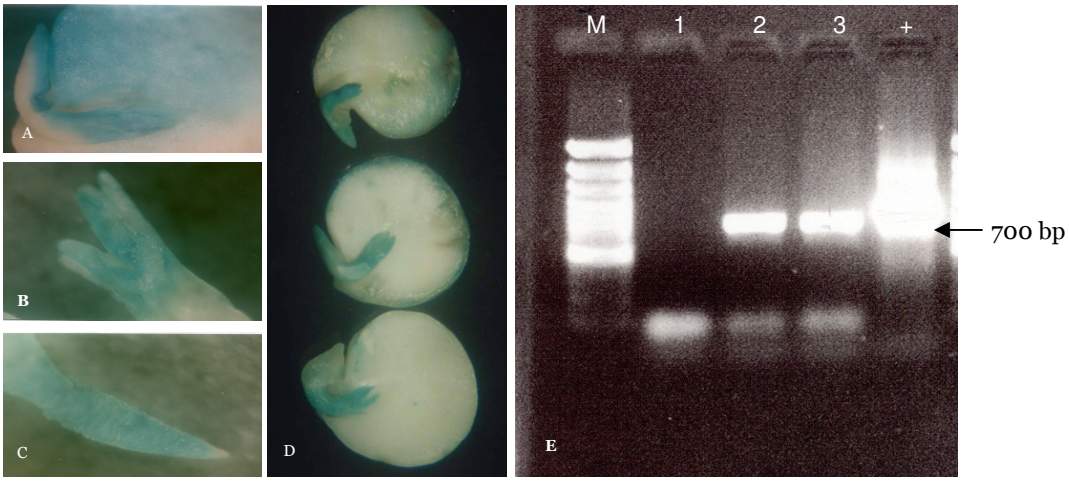


Fig. 19: Transient GUS expression in lentil embryos transformed with LBA4404 pBI121 (left, A-D) and PCR amplification for GUS gene in T_0 plants (right, E where M=100bp marker, 1,2,3 = T_0 lentil samples and + = positive control).

Genomic DNA from transformed T_0 plants were run in a PCR and a product size of about 700 bp was obtained corresponding to the integration of GUS gene (Fig 19).

Different explants demonstrated varying frequency of infection; embryos with single cotyledon disc were best among the lot with a 94 - 100% transient GUS expression followed by decapitated embryo with 80 -100% while cotyledonary nodes were showing 60 -78% response (Fig 20 A). The batches contained 50 explants of each type. The experiment was replicated 4 times.

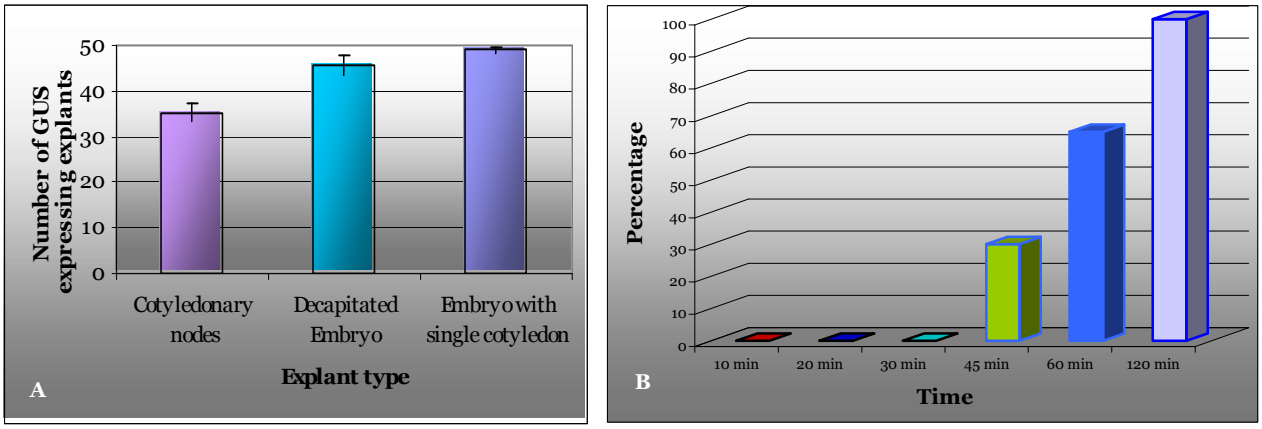


Fig. 20: (A) Transient GUS expression of different explants (B) Optimization of incubation period

Along with transient GUS assay the incubation time in bacterial suspension and infection period for transformation work was tried to be optimized. 25 CE explants were treated for each time

period and expression of the reporter gene was checked through transient GUS assay. It was found that the frequency of infection was higher if the incubation period is longer than 45 min (Fig 20 B). A longer co-culture period (4-5 days), however, provided more potential infected shoots, but this was not feasible as after this log period always bacterial overgrowth which could not be controlled hence the co-culture period was optimized at 3 days.

5.4 Transformation with pSCP1 construct

Based on the results from transient GUS assays, the following experiments were continued with the plasmid construct pSCP1 (harbouring *Ri-pgip* gene) which is harboured inside EHA 105 pSoup. The gene (*Ri-pgip*) confers resistance to fungal infections (Toubart, 1992) As *Agrobacterium* infects through wound sites, the cut surface area was increased by longitudinal slicing of the embryo with single cotyledon disc decapitated at the shoot end, and a second modification by drying the explants for 45-60 min after preparation.

After transformation, the *in vitro* grown explants were observed to grow nicely with elongated shoot, roots and leaves. By the 3rd day after co-culture and transfer to MS media without selection, the explants were growing into healthy seedlings. After applying selection pressure at 2.5 mg/l PPT in the media the growth was not suppressed. An average of 77.38% explants survival has been observed before increasing the selection pressure to 5 mg/l PPT in the culture media. This was done at 12 – 14 days interval. Increase in the selection level affected the plant growth drastically by lowering the survival rate to 18.37% before transferring them to the next higher selection level. It should be mentioned here that the first shoot and the dying leaves (if any) were removed to make sure the growth of the truly transformed shoots in the higher selection media. It was observed that this attempt became lethal to the explants somehow and resulted in lower survival frequency. The next selection pressure used was 7.5 mg/l PPT, after this pressure only few explants can be transferred to 10 mg/l PPT selection, but they could not be saved. It seems like they cannot withstand the higher selection pressure, probably because the root part of the prepared explants were not transformed or the lentil cells are too sensitive to selection pressure (Table 31). The gradual increase in the PPT was done firstly to avoid selection shock for the plantlets and secondly to minimize any escapes or chimeras as generally there always tend to be some escapes in transformation work. A set of control explants were always maintained to check the range of selection pressure. It has been observed that at 2.5mg/l PPT all the control explants died within 10 - 14 days. So this selection level was taken as initial benchmark to screen putative transformed explants.

12 plantlets surviving after 5 mg/l PPT selection pressure were transferred to Seramis containing pots but their growth was far too poor than that of plantlets grown on media without selection. Bud formation and flowering were also observed in these plants, but they were either shedding off or dying afterwards. One seed from T_0 plant 9-5 was collected but the seed did not germinate as it was deformed/dry though the pod was normal looking from outside.

The transformation experiment was also carried out without selection pressure in the media. The survival rate of explants is 91-95% (data not shown) in this case. Since cultured on media devoid of PPT, the explant's growth were vigorous compared to the ones grown on selection media and less death occurred. Some of these plants were transferred to either soil or Seramis containing pots and are found to be growing well just as normal plants (Table 31). Usually the plants started to flower by 5 - 8 weeks after transferring to soil, and afterwards pod formation and seeds were formed.

Explant type	Exp no.	No. of Explants	Use of selection	Survivors 2.5 mg/l PPT	Survivors 5 mg/l PPT	Survivors 7.5 mg/l PPT	Transfer Soil/Seramis	Survivors
LSDCE*	Ex 1 - 10	1146	Yes	1016	86	12	12	9
	Ex 11-16	406	No				260	175
	Ex 17-20 (SMF)	162	No					Under process

* LSDCE = Longitudinal slice of the embryo with single cotyledon disc decapitated at the shoot end

Table 31: Relative response of the lentil explants towards selection pressure.

5.4.1 Molecular analysis of the plants

5.4.1.1 Analysis of *Ri-pgip* clones

Batches were prepared out off the T_0 plants for each experiment. Each batch consisted of 4-5 plants in it. Leaf samples of the plants were subjected to DNA isolation followed by PCR run for HMG, *bar* and *Ri-pgip* (GOI) genes. The 9 surviving plants from Ex 1 to 10 cannot be characterized as leaf material collection was not possible for very poor health condition of the plants. From 6 transformation experiments (Ex 11-16) a total of 175 survived out of 260 plants transferred to pot and they were analysed at the molecular level. 87 of these T_0 plants were PCR positive for the gene of interest (Table. 32). A mean initial transformation frequency was calculated about 29.06 ±6.02 (SE).

Ex no.	Total no of explant	Total plants transferred to soil /Seramis	Survivors T_0	PCR +ve plants (GOI)	% of Transformation
Ex 11	35	34	31	12	34.28%
Ex 12	58	38	32	26	44.82%
Ex 13	106	22	17	9	8.49%
Ex 14	107	70	65	27	25.23%
Ex 15	60	56	-	-	Batch lost
Ex 16	40	40	30	13	32.5%

Table 32: Transformation efficiency selection free cultures from pSCP1 –*bar, Ri-pgip* construct with T_0.

In order to make the PCR analysis more effective, batch PCR was used. Fig 21 is showing the method and the result of batch PCR of one of our transformation experiments (ex-14). From a total of 65 plants, 17 batches were made for selection and 13 of the batches were positive for *pgip* and *bar*. 27 individual were positive with the PCR out of 35 total plants (some plants had died and could not be analysed). The plants in the pots matured and formed T_1 seeds.

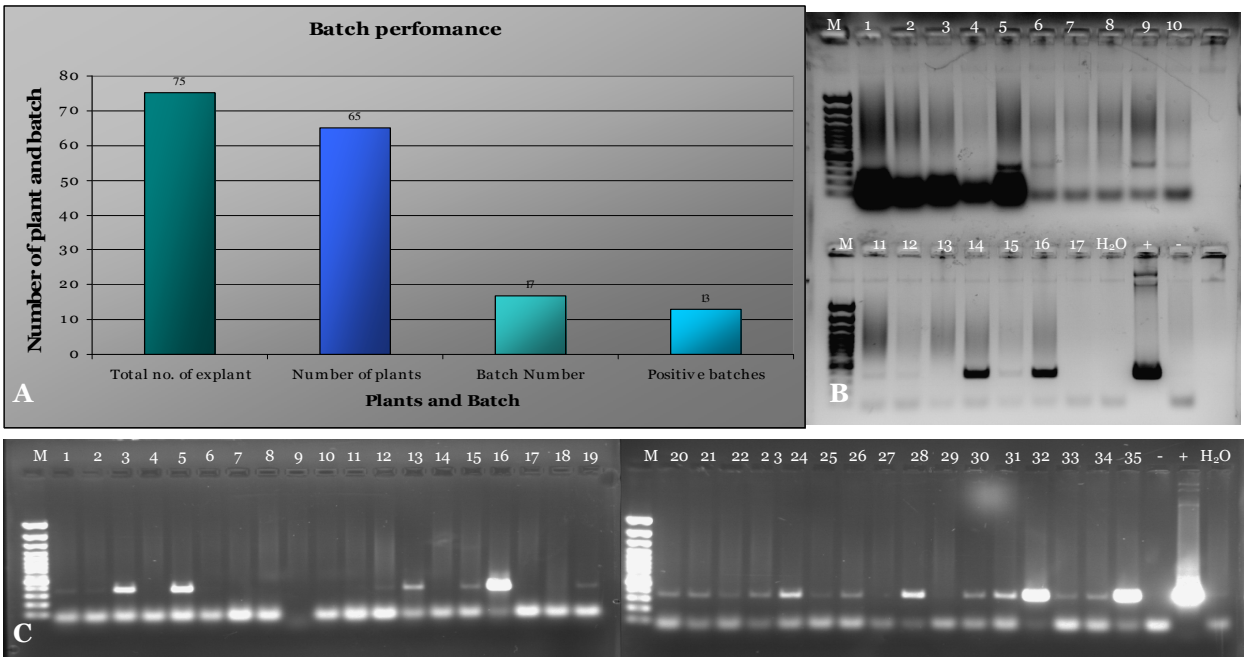


Fig. 21 (A) Performance of Batch (Ex 14) (B) Batch PCR of Expt. no.14 for *Ri-pgip* (C) *Ri-pgip* PCR for individuals from the positive batches from expt-14.

Transformation experiment 14 was selected for further intense analysis. So far 85 seeds (pSCP1) from T_o plants have been planted in the greenhouse along with controls (non-transformed). The controls were used to compare the growth and were source for negative control. It was found that branching in T_n (n= number denoting progeny) was comparatively low, resulting in less leaf material. Otherwise no significant differences were noticed. The T_1 seeds germinated and gave rise to T_1 plants.

DNA isolation and PCR run for *Ri-pgip* and *bar* gene were done from the T_1 plants. Figure 22 is showing the results of PCR run of some of the plants (siblings) from three T_1 clones of experiment 14 for *Ri-pgip* gene (365 bp) and *bar* gene (452 bp). HMG PCR was also run for lentil for the said clones to check DNA quality.

The progeny of 17 T_o clones out of 4 different transformation experiments were grown in the greenhouse to analyze the T_1 plants. 71 out of 106 T_1 plants were positive for the GOI in the PCR run (Table 33).

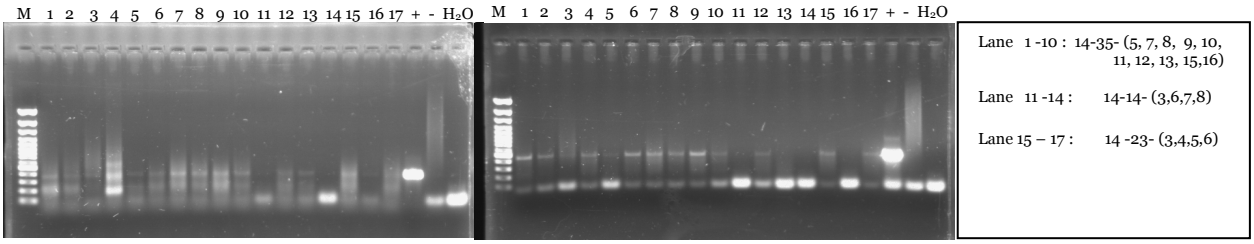
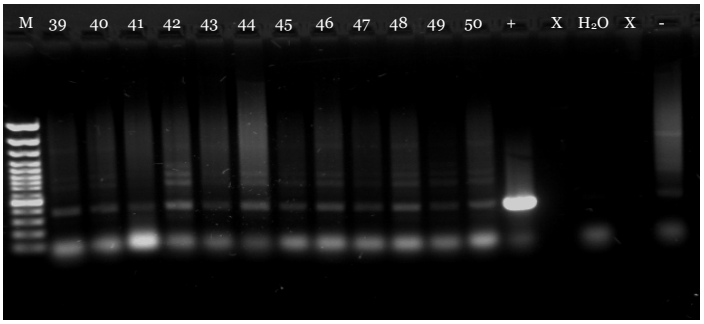


Fig. 22: PCR run for different T_1 plants (Ex 14) and their siblings for (A) *Ri-pgip* and (B) *bar* gene inheritance.

T_2 seeds were obtained from the T_1 plants, some T_2 seeds were selected and were grown in the green house and molecular characterization was done from the surviving clones. T_3 seeds are collected for further molecular characterization.

T_o	T_1 Plant (survivor)	Total plants	PCR +ve *Ri-pgip*	*Bar*	HMG	Functional assay Y/N
11 -16	11-16- 1	1	1	1	1	Y
11-20	11-20- 1	1	1	1	1	Y
11-21	11-21- 1	1	1	1	1	Y
11-23	11-23- 1	1	-	-	1	N
11-29	11-29- 1	1	-	-	1	N
12 -4	12-4- 1,2,3	3	2	3	3	N
12-6	12-6- 1,2,3,4,5,6	6	5	5	5	N
13-5	13-5- 1,2,3,4,5	5	?			N
13-13	13-13- 1,2,3,4,5	5	?			N
14-6	14-6- 1,2,3,4	4	3	3	3	Y
14-14	14-14- 1,2,3 ... 14	14	13	13	12	Y
14-15	14-15- 1,2,3 ... 18	18	18	18	17	Y
14-23	14-23- 1,2,3 ... 12	12	3	2	2	Y
14-35	14-35- 1,2,3 ... 17	17	17	17	17	Y
14-43	14-43- 1,2,3 ...6	6	1			Y
14-53	14-53-1,2,3 ...7	7	6	4	6	Y
14-64	14-64- 1,2,3,4	4	?			Y

Table 33: Analysis of T_1



Lane 43 : 14-14-8-1

Lane 44-45 : 14-35- 5- (1,3)
Lane 46-47 : 14-35-8- (1,2)
Lane 48 : 14-35-9-1
Lane 49 : 14-35-11-1
Lane 50 : 14-35-12-1

Fig. 23: *Ri–pgip* PCR for T_2 plants of 2 T_1 clones of ex 14.

T_1 plants from selected seeds of T_0 clones 14 were analysed at the molecular level (Fig. 23). 49 individual plants from the same clone came positive with 15 in the PCR run for *Ri-pgip* gene. Table 34 shows the results of T_2 plants of the present investigation.

T_0 Clone	PCR	T_1 plant	PCR	T_2 plant	Total plant	PCR +ve (GOI)	PG Assay	T_3 Plant	Total plant	PCR +ve (GOI)
14-14	+	14-14- 8	+	14-14-8-1	1	1	Y	x		
14-15	+	14-15- 14,18	+	14-15-14-1 ...10	10	-	Y	x		
				14-15-18-1 ...10	10	-	Y	x		
14-23	+	14-23-1	+	14-23-1-1 ...4	4	?	N	x		
14-35	+	14-35-5,8,9,12,15	+	14-35-5-1 ...3	3	2	Y	14-35-5-3-1 ...8	8	8
				14-35-8-1,2	2	2	Y	x		
				14-35-9-1	1	1	Y	x		
				14-35-11-1	1	1	Y	x		
				14-35-12-1 ...3	3	3	Y	x		
				14-35-15-1,2	2	-	Y	x		
14-43	+	14-43- 1	+	14-43-1-1 ... 4	4	?	N			
14-53	+	14-53-1	+	14-53-6-1 ...8	8	5	N			

Table 34: Analysis of T_2 and T_3

The T_2 plants provided seeds. Analyses of T_3 plants were done only in a limited number. Clone 14-35 was chosen and 8 T_3 plants of this clone were grown in the greenhouse to be analysed. All of these 8 plants were positive in the PCR run for our GOI. Results are shown in table 34. From this result it points at there was no segregation, probably was leading to a homozygous line.

Furthermore, with this new method of non selection and batch PCR, the tissue culture step is minimized, the process is faster and somaclonal variation may also be minimized. Moreover, it can be hypothesized here that once herbicide resistant transgenics are produced in this adopted method, it will be easier to produce marker free transgenics too.

5.4.1.2 Backbone analysis

Agrobacterium - mediated gene transfer is not always following the textbooks. In some case not only the T-DNA from the Ti plasmid is transferred but also plasmid DNA beyond the left border. As the pSCP1 vector used for transformation contains an *nptII* gene in its backbone, amplifying that region will report us about presence of that particular sequence part in the template. To find out the possible transfer of backbone into the putative clones, kanamycin backbone analysis was done through another PCR run. A schematic representation of the backbone is showed in fig 24 A. The subjected T_1 plants from experiment 11, 12 and 14 came out negative i.e. there were no bands responsible for presence of any sequence from vector backbone confirming that there was no undesirable vector backbone insertion in the gDNA of the clones.

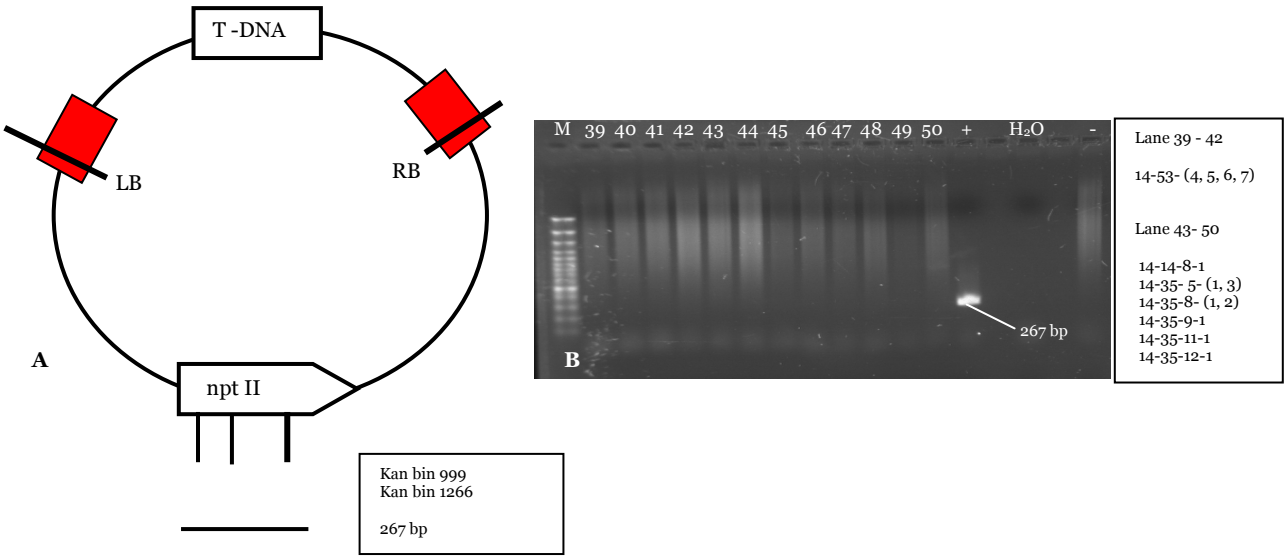


Fig. 24: (A) Schematic diagram of the Backbone (Richter, 2004) (B) Backbone analysis with kan bin 999/1266 primers (see table 14) of plants from T_1 (lane 39-49) and T_2 (lane 43-50).

In figure 24B represents part of the results of the backbone analysis. The T_1 clones of Ex 14 were tested for presence of vector backbone sequence and they were found clean of backbone contamination from the vector. Similar result was obtained with T_2 clones from the same experiment where the DNA samples from the plant that were positive with *Ri-pgip* were tested with Kan bin primers for detection of any undesired presence of vector backbone.

5.5 Functional Analysis of the plants

5.5.1 Leaf paint assay of the lentil clones

Leaf paint assay was also carried out with the commercial herbicide BASTA® at different dilutions on some plants from T_1 and T_2. Leaf paint analysis provides evidence whether the level of *bar* expression and PAT enzyme activity is sufficient to confer resistance to BASTA®. It is a contact herbicide and it will affect only the treated part, as BASTA® is not transported throughout the plant. A pair of leaflets was chosen from the transformed and also from the non-transformed lentil plants growing in the green house for the test. One leaflet of each pair from transformed and a non-transformed control lentil plants were treated with 37.5, 75, 150, 300, and 600 mg/l BASTA® using a small paint brush and leaving the alternate leaflet untreated as control, to optimize the BASTA® concentration for application on lentil (see 4.3.9.5).

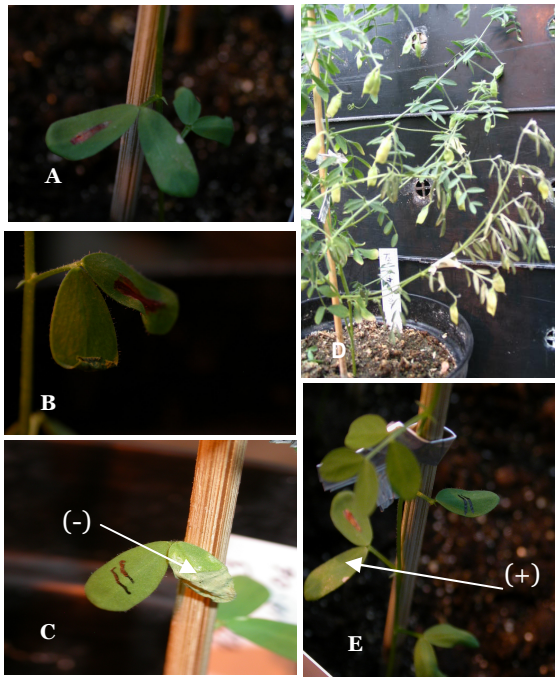


Fig. 25: Leaf paint assay of lentil, 7 days after BASTA® application. A = transgenic leaf treated with 37.5 mg/l BASTA (+); B= 150 mg/l treatment (+/-); C= Transgenic leaf treated with 300mg/l BASTA; D= branch of putative transgenics treated with 600mg/l BASTA; E= transgenic plant showing different BASTA® concentration (300 mg/l = -ve, 37.5 mg/l = +ve) effect on lentil.

As a concentration of 600 mg/l was set for the pea plants as level for resistance against BASTA® in our lab before, this concentration was taken initially as level for the lentils.
But this concentration turned out to be lethal as lentils are very sensitive to herbicides. A good number of plants were lost due to high application of BASTA®. 37.5 mg/l was set as maximum limit of BASTA® concentration to be used for leaf paint in our study.

The effect of treatment was observed after 2-3 days on non-transformed plants where the treated leaflets start wilting. The final evaluation was done after one week of the treatment; it became clear as the treated leaves of the non-transformed plants showed necrotic symptoms

turning brown afterwards and died. On the other hand, the leaves from the transgenic plants were unchanged and stayed green and healthy thus showed tolerance to BASTA® application (Fig. 25). The symptoms were same as the untreated leaflet of non-transformed negative control plant, which were left as internal control for the treatment.

Ri-pgip and *bar* genes are closely linked on the T-DNA, although the clones were positive in the PCR for *Ri-pgip* and *bar* but not all of them were showing resistance to BASTA® (Table 35). For example, the T_1 progeny of clone 14-35, and 14-14 which were proved positive in PCR using *Ri-pgip* and *bar* primers, had negative leaf paint results. This may be due to an unexpressed *bar* gene, but 14-23, 14- 43, 14 -64 were positive in the test. Overall from total 65 T_1 and 28 T_2 plants (ex 11 to 14) positive leaf paint resulted from 34 and 12 respectively.

Generation T_1	PCR	Leaf paint 37.5 mg/l	seed	Generation T_2	PCR	Leaf paint 37.5 mg/l
14-14-8	+	-		14 -14-8-1	+	-
14-35-5	+	-	3	14 -35-5-1	+	-
				14 -35-5-3	+	-
14-35-8	+	-	2	14 -35-8-1	+	-
				14 -35-8-2	+	-
14-35-9	+	-	1	14 -35-9-1	+	-
14-35-11	+	-	1	14 -35-11-1	+	-
14-35-12	+	-	1	14 -35-12-1	+	-

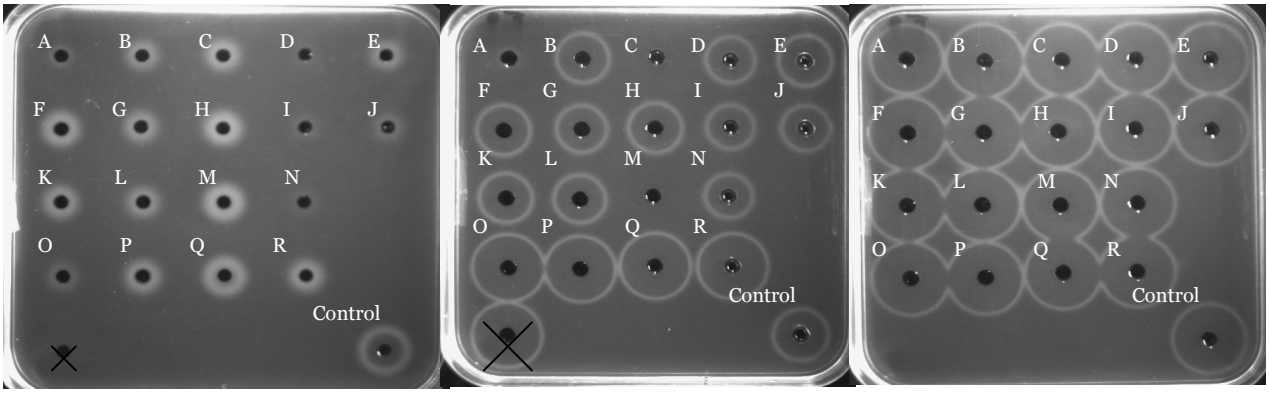
Table 35: Responses of transgenic lentil generations in Leaf paint assay with BASTA®

5.5.2 Expression analysis of the *Ri-pgip* gene materials Polygalacturonase-inhibition assay

To find out the expression or activity of the inserted *Ri-pgip* gene, PG assays were done (see 4.3.9.1). As the functional test to prove the activity of the polygalacturonase-inhibitory protein from raspberry, a PG inhibition test (Taylor and Secor, 1988) was carried out. In addition raw extracts of the transgenic plants and the suitable non transgenic control plants were prepared and their effect was tested for different fungal polygalacturonases.

To test the expression of *Ri-pgip* within a line, raw extracts were extracted from leaves of 18 different individual plants from T_1. In each case, 20 µg (20,000 ng) total protein was used for the PG inhibition test. The raw extracts were tested against polygalacturonases from

Colletotrichum acutatum, *C. lupini* and *Botrytis cinerea*. In each case the halo marked with 'PG' is the endogenous polygalacturonase from the fungal raw extracts and the halo marked with 'Control'. is the raw extract of the non-transgenic control plant with suitable fungal polygalacturonase. The extracts of the transgenic plants that are to test are in the other marked halos.



Botrytis cinerea *Colletotrichum lupini* *Colletotrichum acutatum*

Fig. 26: Different inhibitory effect against different fungal PG tested (here, in holes 'A-R' contain total protein from different T_1 plants and 'Control' is the non transgenic plant, tested against 'PG' from *B. cinerea , C. lupini and C. acutatum*).

Some plants from the T_1 and T_2 of Ex 14 showed significant responses against different fungal PGs (*Botrytis cinerea, Colletotrichum lupini, C. acutatum*). Fig 26 is showing the positive responses from some T_1 plants from 14-35, 14-14, 14-23 in their PG assay.

The clones were not showing any significant response against *C. acutatum*. In the figure, it is visible that the raw extracts of the individual T_1 plants had differently strong effects on the polygalacturonase of *Colletotrichum lupini* and *Botrytis cinerea*. The leaf extracts of the plants had an inhibition activity on *Colletotrichum lupini* with an average 48.26% and 52.77% for *B. cinerea*. The same leaf extracts exhibited no effect on polygalacturonases from *C. acutatum* (Fig. 26). The raw extract of the non transgenic plant had almost no inhibition against the fungal polygalacturonases.

A total of 68 T_1 plants were subjected to PGIP assays. More or less all the plants showed varied level of inhibition including the non-transformed control lentil plant as it was also grown in the greenhouse together with the other plants. It was observed that the transgenic plants were more effectively inhibiting the PG from *B. cinerea*, by showing an inhibition ranging from lowest 3.52 to highest 100% and an average of 49%. On the other hand the same plants were showing comparatively less effectiveness in general against *C. lupini* but average inhibition being the same (Table 36).

Generation	Clone number	PCR	Leaf paint	% of Inhibition for		
				B. cinerea	*C. lupini*	*C. acutatum*
T_1	14-15-1	+	-	62.55	40.47	0
	14 -15-2	+	-	66.23	33.09	0
	14 -15-3	+	-	35.23	33.49	0
	14 -15-4	+	-	x	x	x
	14 -15-5	+	-	70.84	70.72	0
	14 -15-6	+	-	62.30	50.36	0
	14 -15-7	+	-	18.41	37.30	0
	14 -15-8	+	-	51.87	52.79	0
	14 -15-9	+	-	69.36	31.46	0
	14 -15-10	+	-	60.46	44.28	0
	14 -15-11	+	-	x	50.36	x
	14 -15-12	+	-	100	37.14	0
	14 -15-13	+	-	63.59	50.20	0
	14 -15-14	+	-	45.24	28.95	0
	14 -15-15	+	-	38.79	38.36	0
	14 -15-16	+	-	50.58	37.87	0
	14 -15-17	+	-	65.00	58.06	0
	14 -15-18	+	-	75.19	100	0

Table 36: Inhibition activity of the putative T_1 transgenics against different PGs.

It was also observed that siblings from one clone were showing different levels of inhibition against same PG. To confirm the previously shown results, 18 individual plants of a progeny were pulled up from 14-15-1 to 14-15-18 (T_1) for the PG inhibition test and their inhibition activity were examined after 3 weeks of germination. Here also 20,000 ng of the raw extract were used.

In fig 27 A and 27 B, individual plants from line 14-15-1 to 18 showing varied activity (expression) of the leaf extracts i.e. the polygalacturonase inhibitory protein against the polygalacturonase of *Colletotrichum lupini* and *Botrytis cinerea*. As observed in the figure, the siblings were effective against *B. cinerea* with an average of 58.46%. On the other side, about 11% performance reduction was shown by the same siblings against *C. lupini*. Notably these plants were also positive in PCR for GOI.

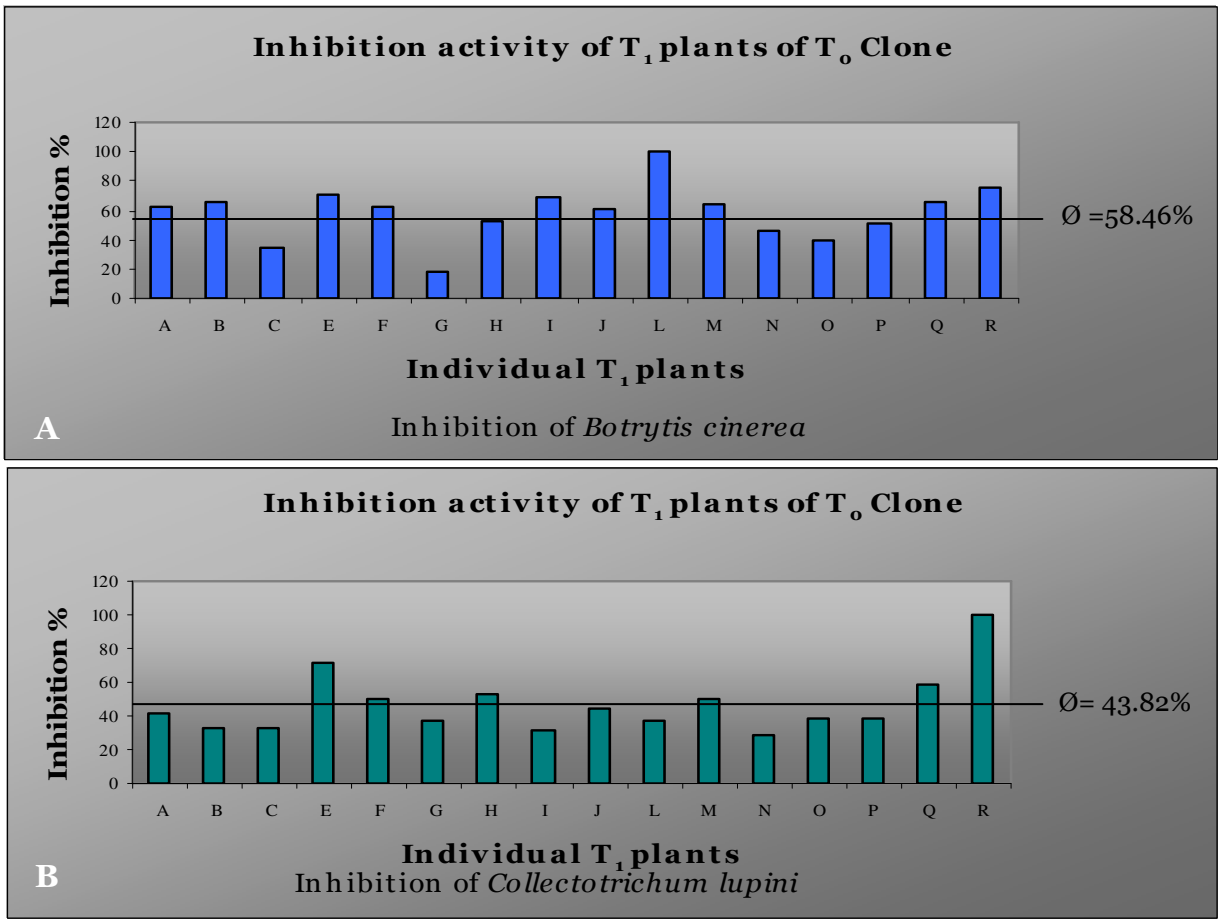
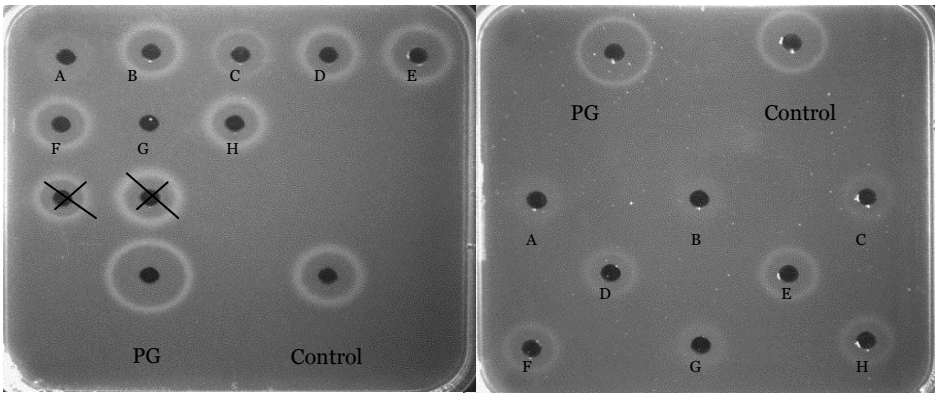


Fig 27: Graphical presentation of varied inhibition performance of T_1 (Ex -14) siblings against different PG Inhibition against *Botrytis cinerea* (B) Inhibition against *Colletotrichum lupini*

Inhibition activity was also found efficiently working by the T_2 plants that were subjected to PGIP assays (Fig.28). Here also the clones were showing varied inhibition responses against the introduced PGs. In case of *Botrytis,* the subjected T_2 plants of Ex 14 had shown lowest 41.37% to highest 67.58% while for *Colletotrichum* the inhibition was lowest 40.95% to highest 56.77%. This response once again proves the stronger activity of the raspberry PGIP against *Botrytis*.

The leaf extracts with increasing age of the plants showed a lower inhibition activity. In the T_1 progeny, 20µg of total protein extract showed inhibitory activity against the above mentioned PGs but showed almost no herbicide resistance. The T_2 plants were also negative in herbicide resistance. Table 37 is showing the responses of the T_2 plants derived from T_0 clone 14-35.The inevitably occurring herbicide sensitivity of the progeny was independent from expression instabilities of the gene of interest.



T_2 samples:

A- 14-14-8-1
B, C- 14-35-5- (1, 3)
D, E- 14-35-8- (1, 2)
F- 14-35-9-1
G- 14-35-11-1
H- 14-35-12-3

Botrytis cinerea *Colletotrichum lupini*

Fig. 28: PG activity of the T_2 plants.

T_1 Clone	PCR	Leaf paint	% of Inhibition for		T_2 Clone	PCR	Leaf paint	% of Inhibition for	
			B. cinerea	*C. lupini*				*B. cinerea*	*C. lupini*
14-14-8	+	-	70.04	82.88	14 -14-8-1	+	-	58.99	56.77
14-35-5	+	-	84-47	80.19	14 -35-5-1	+	-	53.28	57.17
					14 -35-5-3	+	-	53.28	48.33
14-35-8	+	-	59.82	84.19	14 -35-8-1	+	-	53.28	48.98
					14 -35-8-2	+	-	41.37	43.39
14-35-9	+	-	87.52	39.83	14 -35-9-1	+	-	54.20	40.95
14-35-11	+	-	56.48	37.40	14 -35-11-1	+	-		47.76
14-35-12	+	-	66.60	33.64	14 -35-12-1	+	-	55.67	44.76

Table 37: Responses of T_2 clones in PG assay.

5.6 Marker free pGreen Vector T- DNA Construction

5.6.1 Cloning of 35s promoter and terminator into PGII OOOO

The pGIIMH35s (5540 bp) contains the *bar* gene between a *nos* promoter and terminator and also contains a double 35s promoter and 35s terminator including a translation enhancer in between. The 35s promoter and terminator cassette (~1150 bp) were excised successfully by digesting the pGII 35s plasmid with *KpnI* and *SacI* (see 2.3.2). On the other side PGII0000 (3304 bp) contains only multiple restriction/cloning sites between its LB and RB and nothing else, making it appropriate to be used as a vector for our work. This plasmid was also digested with *KpnI* and *SacI* to prepare the vector which is to contain the 35s cassette from PGII35s. Both of the digested products were run in an agarose gel and the proper bands were excised from the gel and clean DNA fragment (see 4.3.3.2) was obtained through GFX column (Fig. 29).

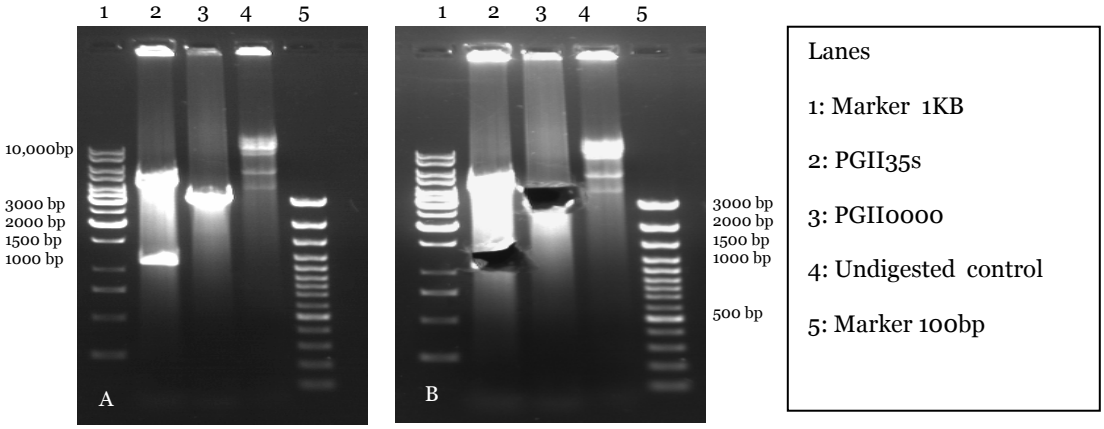


Fig. 29: (A) Results of agarose gel electrophoresis after restriction digestion of PGII0000 and PGIIMH35s with *KpnI* and *SacI*. (B) The gel after band excision.

The resultant plasmid DNA fragments were subjected to ligation (see 4.3.5) in a 1:3 vector insert ratio to construct the basic PGII vector. Competent *E. coli* cells were transformed (see 4.2.3) with the ligation mix, spread on LB plates supplemented with kanamycin in different aliquots and grown overnight at 37°C. Next day there were small *E. coli* colonies on the plates. Colony PCR was done with the resultant colonies with pGreen primers 297(forward) and 303(reverse) to select the positive colonies. The expected band after the PCR reaction was ~1158 bp if the ligation worked and 250 bp if it failed. Band of the right size was obtained in the colony PCR (Fig. 30).

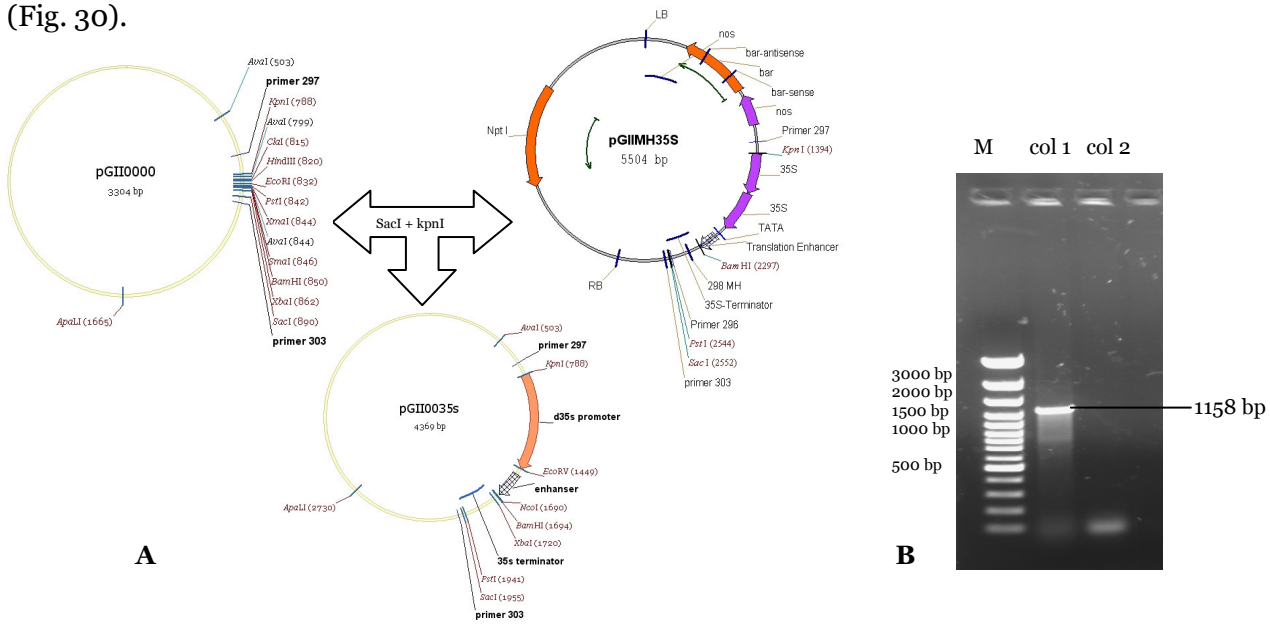


Fig. 30: (A) Resultant PGII0035s by ligation of parts from PGII0000 and PGIIMH35s
(B) Colony PCR of the *E. coli* colonies after transformation with ligation mix.

The positive colony from the PCR was selected for further confirmation of the proper ligation. Plasmid DNA was isolated from the overnight culture and restriction digestion was carried out

for confirmation test. Digestion with *KpnI* and *SacI* gave two fragments, a smaller one of 1158 bp for the insert and a bigger one 3304 bp for vector backbone. *BamHI* digestion was to give only a single band (~4369 bp) as there is only one *BamHI* site in the vector, digestion with it is to linearize the vector only and no further fragments (Fig. 31) appear.

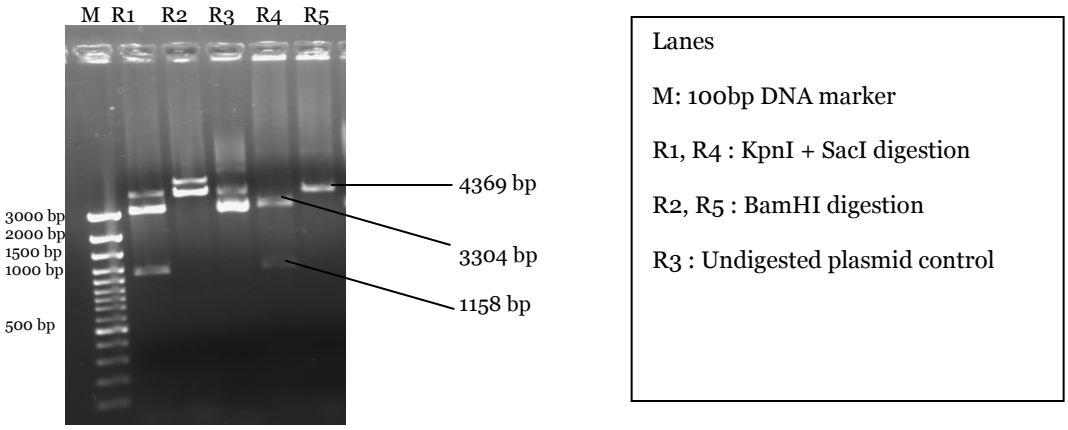


Fig. 31: Restriction digestion of the plasmid DNA isolated from *E. coli* after transformation.

5.6.2 *Ri-pgip* insert preparation for cloning through PCR

To clone the *Ri-pgip* gene into the prepared PGII vector, the GOI insert was prepared through PCR using combizym polymerase and amplifying pSCP1 plasmid containing the *Ri-pgip* gene with modified pSCP1 *BamHI* forward and reverse primers since the cloning is to be made at the *BamHI* site of the vector (see table 17, 18). The annealing was done better at 65°C (Fig. 32). A product about 998 bp in size was expected and was obtained. The PCR product was cleaned through GFX column for use in cloning (see 4.3.3.1).

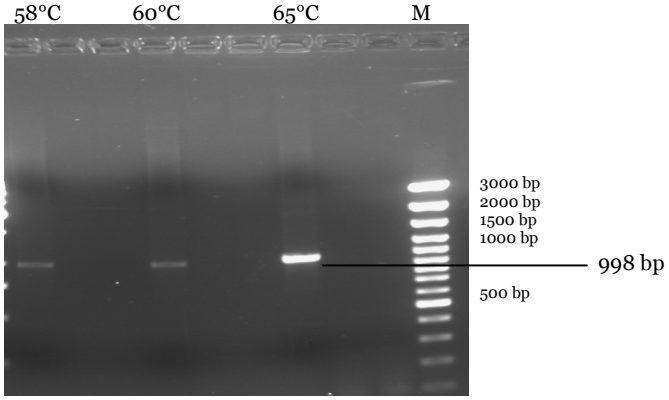


Fig. 32: *Ri-pgip* insert preparation for cloning.

5.6.3 Cloning of the *Ri-pgip* gene in pGEM vector

As the basic pGreen vector without the *bar* gene was successfully obtained, the next step for the cloning work is to put this two parts together. But for some unexplainable reasons these two fragments failed to ligate even after all possible trouble shooting was done. Hence the help of a helper vector pGEM was used in order to succeed. As mentioned before pGEM has the T overhang which makes it easier for the PCR amplified clone product to be ligated. The positive colonies were selected through blue white colony selection (see 4.3.6) by adding 2% IPTG and X-gal in the LB media plates with ampicillin (Fig. 33). Colony PCR was carried out with the positive colonies for further selection.

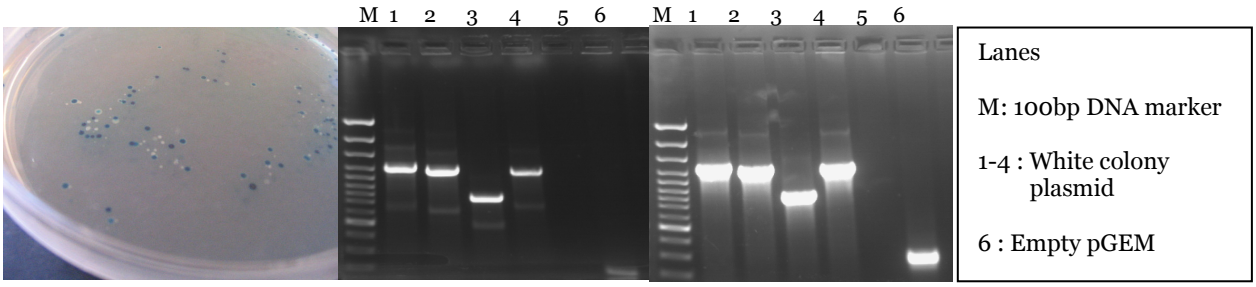


Fig. 33: Blue white colony selection and PCR of the plasmids isolated from the white colonies with primers for pGEM, first SP6, T7 and latter M13 (f & r)

Plasmids were isolated from the colony PCR positive colonies and reconfirmed with PCR with specific pGEM primers SP6, T7 and M13 forward and reverse. In both cases plasmids with an exact insert should produce a product of 1200 bp and 200 bp if empty. The products achieved as expected with the colonies. There were exceptions in some of the colony products which were about 800bp after PCR run; this may be due to partial incorporation of the gene. But such colonies were discarded. The positive colonies were further confirmed through restriction digestion of the isolated plasmids with *BamHI* and *NotI* separately where two fragments in size of ~3000bp and ~1000bp were expected in both cases; and insertion of the gene was confirmed. Again, the plasmid integrity was checked by sequencing of pGEM derived plasmids. The results of BLAST search at NCBI database using the sequencing results of constructed plasmid found 99% homology to PGIP gene from *Rubus idaeus* L. (Fig. 34).

>gi|40732889|emb|AJ620336.1| *Rubus idaeus* mRNA for putative polygalacturonase-inhibiting protein
(*pgip1* gene)
Length=1254

Score = 987 bits (498), Expect = 0.0
Identities = 501/502 (99%), Gaps = 0/502 (0%)
Strand=Plus/Minus

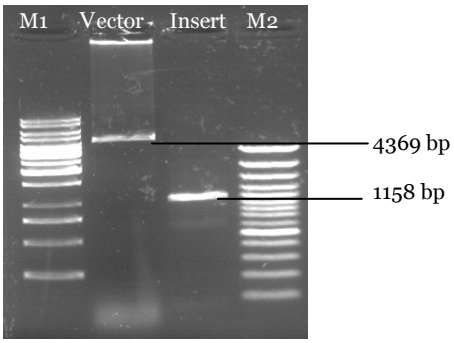
```
Query  23   TTACTTGCAACTTGGGAGGGGAGCACCGCAAAGGCACCGGTTATGGAAATACGACGCGGT  82
            ||| ||||||||  |||| ||||  || |||  ||||  |||||  ||||  |||  ||||||  ||||  |||||  ||   | ||
Sbjct  1024 TTACTTGCAACTTGGGAGGGGAGCACCGCAAAGGCACCGGTTATGGAAATACGACGTGGT  965

Query  83   GTCCAAGCTCTGCAACTTCCCACCCACCGGAATCTTACCACACAACCTGTTGTAGCTCAC  142
            ||  ||||  ||||||  ||||||||  ||  ||||||  ||||  ||||||||||  ||||||  ||||||  |||  |||||
Sbjct  964   GTCCAAGCTCTGCAACTTCCCACCCACCGGAATCTTACCACACAACCTGTTGTAGCTCAC  905
Query  143  ATTGAACAACACCAAATCATCCAATTGGGTCAACTGTGCCGGAATACTCCCCGTGATGCT  202
            | |||  |||||  ||||||  ||||||||||  ||||  ||  |||||||||  |||  |||||  ||  |||||  |||||  ||
Sbjct  904   ATTGAACAACACCAAATCATCCAATTGGGTCAACTGTGCCGGAATACTCCCCGTGATGCT  845

Query  203  GTTATGGTTCAAGTCCACGGCTCTCAAGCTGGTCGAAAACACCACTTTGGACAGATCAAA  262
            ||||||  |||  ||||||  |||||  |||||||||  ||||  ||||  |||  ||||||||  |||  |  ||||  |||||||
Sbjct  844   GTTATGGTTCAAGTCCACGGCTCTCAAGCTGGTCGAAAACACCACTTTGGACAGATCAAA  785

Query  263  TTCCAGCATGTTCCTCGACAGATCCACAATCTGGGTGGTCTTGTTCAAACCGAATATTAC  322
            ||||||  |||||||||  ||  |||||  |||  ||||||||  ||  ||  |||  |||||||||  |||  |||||  |||  ||||
Sbjct  784   TTCCAGCATGTTCCTCGACAGATCCACAATCTGGGTGGTCTTGTTCAAACCGAATATTAC  725

Query  323  AGACGCGTCGCCTTCGAGCTTGTTGCGTGACAAGTCTATTTGGTCGAAGTTCATGTTAGC  382
            | ||||  ||  |||  ||||||||  |  ||||||||  |||||||  |||  ||||||||||  ||||||  ||  ||||||||||  ||
Sbjct  724   AGACGCGTCGCCTTCGAGCTTGTTGCGTGACAAGTCTATTTGGTCGAAGTTCATGTTAGC  665

Query  383  AAATGAGGTTGGGATTTTTCCTGTGAGCTGGTTGTGGGAGAGGAAGAGAGCCGGAACGGT  442
            | ||||  ||  |||||  |||||  ||  |||||||  ||  |||||||  |||  |||  |||  ||||  |||  |||  |||  |||||
Sbjct  664   AAATGAGGTTGGGATTTTTCCTGTGAGCTGGTTGTGGGAGAGGAAGAGAGCCGGAACGGT  605

Query  443  GCCAACGAATTTTCCGAATGAGCTAGGAATTTGACCTGTGAGCTGGTTGCGATCTAGATG  502
            |||||  ||||  |||||||||  ||||  ||||  ||||  |||||||  ||  ||||  |||  ||  |||  ||||  |||||||  |
Sbjct  604   GCCAACGAATTTTCCGAATGAGCTAGGAATTTGACCTGTGAGCTGGTTGCGATCTAGATG  545

Query  503  AAGGGCTCCCAAGTTGGGTAGC  524
            ||||  ||  ||  |||||||||  |  |||||
Sbjct  544   AAGGGCTCCCAAGTTGGGTAGC  523
```

Sequences producing significant alignments:	Score (Bits)	E Value
gi|40732889|emb|AJ620336.1| Rubus idaeus mRNA for putative po...	987	0.0
gi|40732908|emb|AJ620355.1| Rubus idaeus partial pgip1 gene f...	672	0.0

Fig. 34: Result of BLAST search at NCBI database for homology of the cloned and sequenced *Ri-pgip* with other organism.

5.6.3 Cloning of the *Ri-pgip* gene in pGII basic vector

As at this stage both the vector and insert were at hand ready to be ligated in order to achieve our desired plasmid construct. Since the *Ri-pgip* gene is flanked by a *BamHI* restriction site, it was imperative to prepare the vector and the insert through *BamHI* digestion. The digested vector and insert product were run in agarose gel and correct gel bands, vector 4369 bp and insert 1158 bp were collected for final ligation (Fig. 35). All the digested vectors and inserts were always cleaned up through GFX column before using them for ligation. Dephosphorylation of the vector DNA fragment was done with SAP (see 4.3.4) to avoid vector self-religation.



Lanes

M1: 1kb DNA marker

2: *BamHI* digested Vector

3: *BamHI* digested Insert

M2: 100 bp DNA marker

Fig. 35: Vector and Insert on agarose gel.

The ligation of the obtained fragments was done with T4 DNA ligase (see 4.3.5) and was transformed into *E. coli* on the next day. The colonies were selected through colony PCR like before and plasmids were isolated from the PCR positive colonies. Only one colony came out positive among 8 randomly selected colonies (Fig. 36 A).

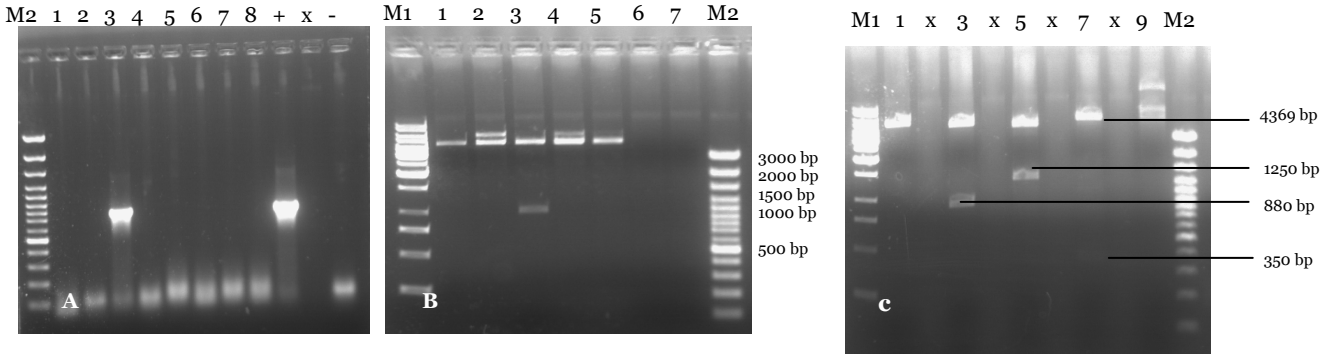


Fig. 36 : (Here, M1 and M2 : 1kb and 100 bp DNA marker , X = Blank)

(A) Colony PCR of colonies (*E. coli* colonies 1 -8, pSCP1 as +ve, PGII0035 as –ve)

(B) BamHI digest of the colonies for selection (Colony 1-7)

(C) Restriction digestion of plasmid DNA of Col. 3 to check the direction of the ligated insert.

The architecture of the constructed plasmids pGIIoo35s -*Ri-pgip* was confirmed by restriction digest initially with *BamHI* for confirmation (36 B) followed by digestion with different restriction enzyme combinations; *Xba*I alone, *HindIII* in combination with *PstI*/*KpnI*/*Nco*I checking of insert ligation direction. The expected fragments for correct direction beside the vector backbone fragment (4369 bp) were 480 bp, 880 bp, 1250 bp, and 350 bp respectively (Fig. 36C) and that was confirmed. The resulting T-DNA is shown in Fig. 37.

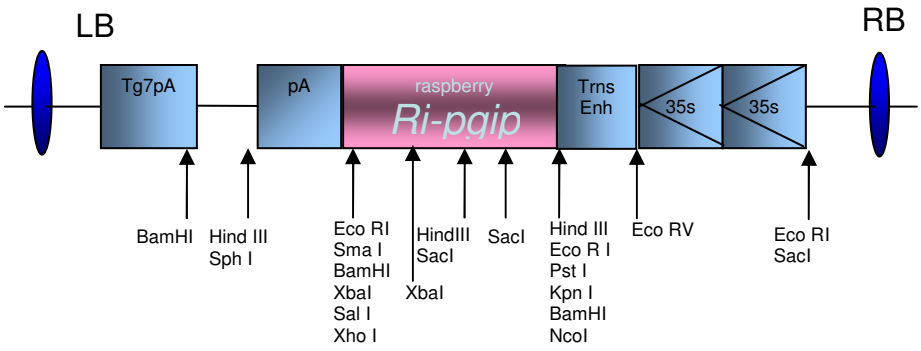


Fig. 37: Schematic diagram of the T-DNA region of the binary vector pGreenII containing r-*Ri-pgip* gene.

The T-DNA region of the binary vector pGreenII is now constructed with respective restriction sites containing the *Ri-pgip* gene with double 35s Promoter, Translation enhancer (Trns Enh), 35s terminator; LB and RB are left and right T-DNA borders and with this denotes the successful integration of the raspberry *pgip* gene into a PGII vector which is free of a selectable marker. The new construct was named PGIIRHoo35s-*Ri-pgip*.

5.6.5 The *Agrobacterium* transformation with PGIIRHoo35s-*Ri-pgip*

To use the *de novo* construct for plant transformation, after confirmation the constructs were transferred (see 4.2.5) into the disarmed *Agrobacterium tumefaciens* strain EHA105 harbouring the pSoup plasmid from the pGreen II collection. The *Agrobacterium* colonies were grown on LB plates supplemented with kanamycin as the construct contains an *npt*I gene in its backbone. In a similar way as previously, colonies were selected through colony PCR, plasmid isolation was carried out from the transformed *Agrobacterium* strain EHA -105 pSoup and retransformation of the isolated plasmid from *Agrobacterium* into *E. coli* was done for final checking.

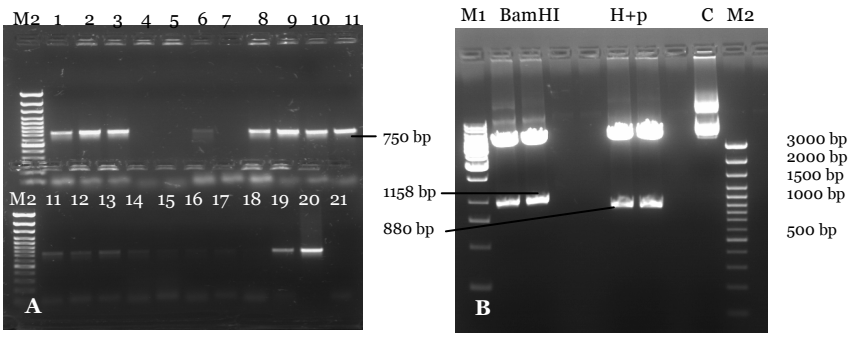


Fig. 38: (Here, M1: 1 kb DNA marker, M2: 100bp DNA Marker, H+P = *HindIII+PstI*, C= undigested control plasmid)

(A) Colony PCR of the transformed *Agrobacterium* colonies

(B) Restriction digestion of the plasmids isolated from retransformed *E. coli*

The *Agrobacterium* colonies were randomly selected for colony PCR with primer (*Ri-pgip* 1,749) for GOI; most of the colonies came out positive. (Fig. 38 A). Some from these colonies were retransformed into *E. coli* and plasmids were isolated. Restriction digestion of the plasmids with *BamHI* and combination of *HindIII* and *PstI* showed the common bigger fragments of 4300 bp and the smaller fragments 1158 bp (*BamHI*) and 880bp (*HindIII+ PstI*) confirming the correct incorporation of the *Ri-pgip* gene and its stability (Fig.38 B).

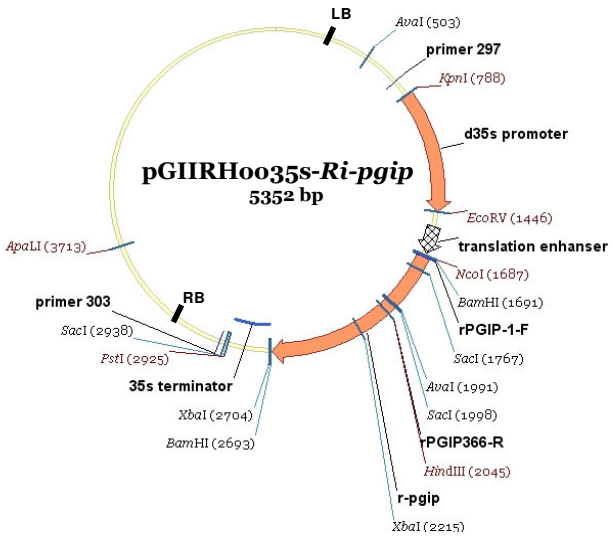


Fig. 39: PGIIRH0035s-*Ri-pgip*; the final cloning product

Figure 39 is showing the functional map of the pGreen vector cloned with the *Ri-pgip* gene. Different glycerol stocks (see 4.2.7) were prepared and stored at -80 °C as deposit for the plant transformation.

5.6.6Transformation of Lentil with PGIIRH0035s-*Ri-pgip*

With the marker free pGreen construct (Fig.39) transformation of lentil explants with this new construct was done. Transformation was carried out in the same manner as mentioned in transformation with pSCP1 construct.

Altogether 4 transformation experiments were carried out (Table 38) with the new construct. These Lentil plants were grown in controlled environment of the growth room. Seeds of the T_0 plants were collected for further analysis.

Ex no.	Total no of explant	Total plants transferred to pot	Survivors	Seed	PCR +ve plants (GOI)	% of Transformation
Ex 17	28	20	10	17	9	28.57
Ex 18	54	24	22	7	11	20.37
Ex 19	45	30	30	8	26	57.77
Ex 20	35	35	35			Under process

Table 38: Marker free transformation with PGIIRH0035s *Ri-pgip*.

5.6.7 Molecular characterization of transformants Detection of T-DNA integration by PCR

The raised plantlets were assessed for positive insertion of the T-DNA from the pGreen vector. Successful integration of the T-DNA into lentil genomic DNA was analyzed using different primers for the *Ri-pgip* gene in the clones of T_0 and following generations by PCR.

Stable integration of T-DNA into genomic DNA of T_0 transformants of lentil was confirmed by PCR using different primer combinations to detect the *Ri-pgip* gene. From a total of 162 explants from 4 transformation experiments, only 46 of them were positive for GOI, the *Ri-pgip* gene PCR.

The results clearly indicate and confirm the successful integration of raspberry *pgip* gene into genomic DNA of transformed lentils. Fig. 39 clearly shows the successful integration of T-DNA into the lentil genome where 26 out of 29 T_0 plants of Ex 19 parts of the *Ri-pgip* sequence could be amplified and produced fragments of an expected product size of 750 bp.

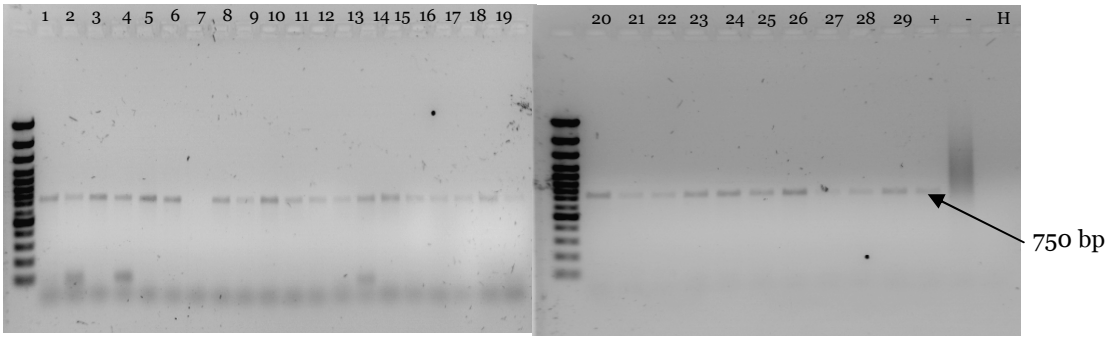


Fig. 39: Marker free T_0 Clones (Ex 19) in PCR for *Ri-pgip* gene.

Table 38 is showing the results from the transformation experiment with the marker free pGreen–*pgip* vector construct. Many clones from different transformation experiments were positive for the GOI presenting unexpectedly higher individual experiment transformation percentage as shown in table 38; the reason for high transformation frequency may have been caused by the chimeras. Or this was may be due to the presence of bacterial sequence, to find out the reason backbone analysis was conducted.

5.6.8 Analysis for Backbone

As PCR may come positive with the GOI primers, specific PCR had to be done to detect presumably residing bacteria between the intercellular spaces of the transformed T_0 plants. It was required to examine the clones for presence of specific bacterial sequences. For detection of any bacteria residing between the intercellular spaces, PCR experiments with PIC-A primers were done to detect the presence of *Agrobacteria*. All except one of the T_0 clones of ex 19 were 'clean' of in the respective PCR run. In very few cases (2 in Ex 17 and 6 in Ex 18) there were positive amplification (~600 bp) from the T_0 clones of the experiment. These clones were discarded for any further analysis.

Possible transfer of vector backbone sequences was also to be checked. This confirmation was done using kanbin 999/ 1266 primers as pGreen vector also contains *nptII* gene in its backbone. According to the results obtained, the T_0 clones of Ex 19 were clean from any backbone sequence contamination.

5.6.9 Analysis of *Ri-pgip* expression in transgenic lentil

For functionally assaying the T_o clones, PGIP assays with different fungal polygalacturonases were done and are under process. A total of 20µg of total protein was used for assay just like previously done in the case of the clones derived with pSCP1 construct transformation.

So far, only PG from *Ascochyta* was used for these newly raised clones. The PG activity of the clones of ex 19 was working quite efficiently against *Ascochyta*. Fig 40 is demonstrating the inhibition activity of T_o clones of ex 19. A total of 21 T_o clones were assayed. More or less all the plants showed inhibition activity. From ex 19, 18 clones out of 21 were inhibiting fungal extract from *Ascochyta*. An inhibition of lowest 0.48% to a highest 80.14% was observed against *Ascochyta*. In fig 41, individual T_o clones of ex 19 showing varied activity of the polygalacturonase inhibitory protein from their leaf extracts against the polygalacturonase from *Ascochyta*.

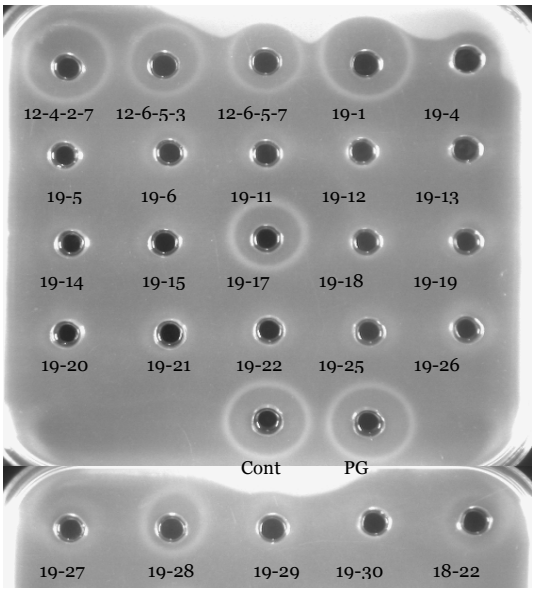


Fig. 40: PG activity of the T_o clones from marker free transformation (ex 19)

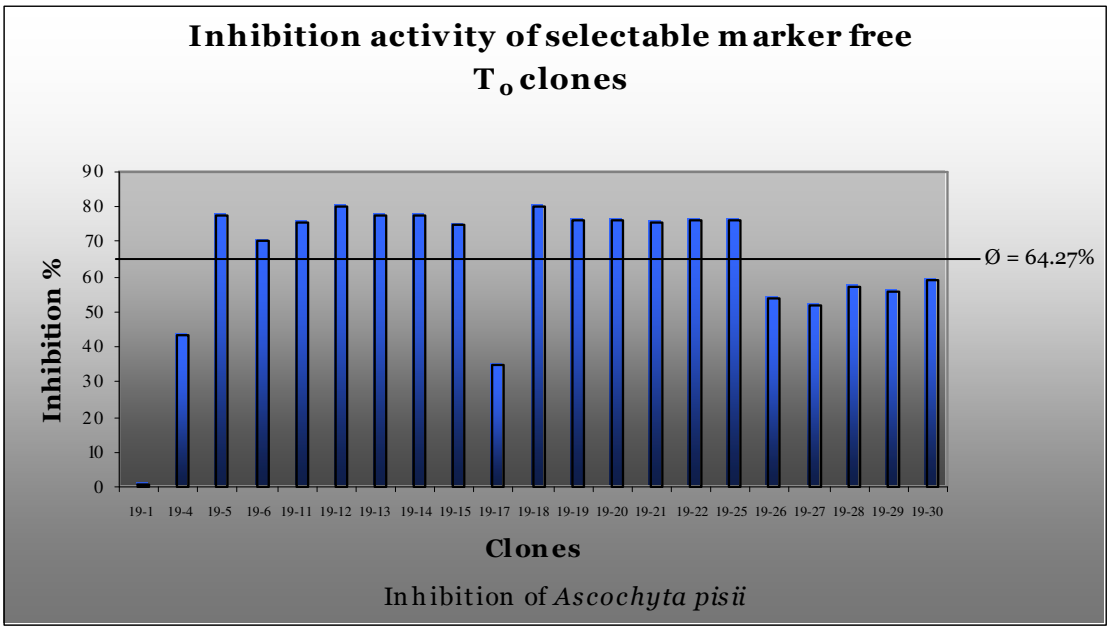


Fig. 41: Inhibition of *Ascochyta* by selectable marker free individual clones of ex 19.

As observed in the figure most of the siblings efficiently inhibited polygalacturonase from *Ascochyta* with an average of 64.27%. Notably these plants were also positive in PCR for GOI. The inhibition activity of the clones is thus demonstrates the proper expression of the inserted *Ri-pgip* gene.

6. DISCUSSION

The present investigation was undertaken to establish an efficient protocol for *in vitro* regeneration of lentil varieties growing in Bangladesh, for suitable *Agrobacterium* mediated genetic transformation and to establish a protocol for a marker free transformation system as well. The study was performed in four phases. In the first phase, *in vitro* regeneration of plantlets has been developed and optimized. The second phase aimed at the genetic transforming capability of various explants of lentil by using genetically engineered *Agrobacterium tumefaciens* strains. The third phase focused on genetic transformation lentil with an *Agrobacterium* strain containing fungus resistant polygalacturonase inhibitory protein gene (*pgip*) from Raspberry (*Rubus idaeus*) and selectable marker gene *bar* (*Streptomyces hygroscopicus*). Fourth and the last phase was targeted to cloning a raspberry *pgip* gene into a pGreen vector without the selectable marker gene *bar* and genetic transformation of lentil was carried out with this construct.

Biotechnological techniques, offer novel possibilities for the transfer of genes between different distant species and thus can be considered as improving conventional plant breeding. A glance on the recent increase of GMO cropping areas will illustrate the success of these technologies including plant transformation and also the varied public acceptance of this new technology. Since initial commercialization in 1996, the global planted area of biotech crops has soared by more than fifty-fold from 1.7 million hectares in six countries to 90 million hectares in 21 countries in 2005. The 8.5 million farmers planting biotech crops in 2005 also marked a significant milestone as the 1 billionth cumulative acre, or 400 millionth hectare, was planted. 1.7 million hectares of the global area of transgenic crops in 1996 increased to 52.6 million hectares in 2001 (3000 %). About 20 % increase was reported between 2003 and 2004. In 2005, four new countries and a quarter million more farmers planted biotech crops as part of an 11 percent increase in global biotech crop area as reported by ISAAA (2006). Both industrial and the developing countries are showing gradual increase in GMO crop production from 1996 to 2005.

All over the world 21 countries so far have adopted the biotech crops (Fig. 42); the mega countries are using 50,000 hectares or more for producing biotech crops. The principal transgenic crops produced were (in descending order) soybean, maize, cotton and canola. In 2005, Herbicide-tolerant soybeans continue to be the most widely adopted trait, accounting for 60 percent of the total global area followed by (21.2 million hectares at 24%), cotton (9.8 million hectares at 11%) and canola (4.6 million hectares at 5% of global biotech crop area). Besides these crops genetically modified (virus resistant) squash, papaya and rice were also grown in

2005. The transgenes introduced into these crops were herbicide tolerance and insect resistance genes. ISAAA projects the global value of the biotech crop market to increase from $5.25 billion in 2005 to $5.5 billion in 2006 (James, 2004, 2005 and 2006).

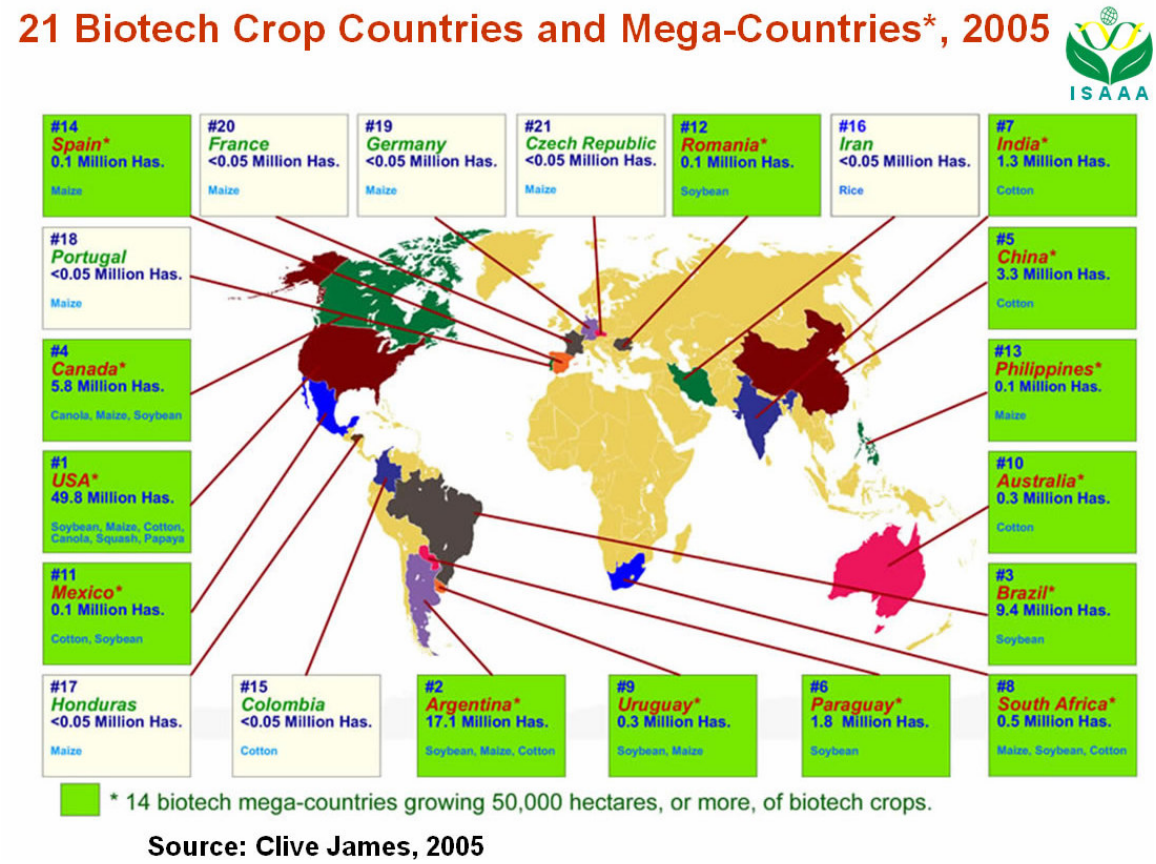


Fig 42: Global area of genetically modified crops, in million hectares in 2005. (Clive James, 2005).

The concentration of the multinational companies were in a way fixed with these GM crops only and most other crops did not get the same interest, particularly grain legumes, which play a major role in the nutrition demand in developing countries. Fungal disease resistance gained less attention although it is an important factor as yield reducer in both developed and developing countries. Producing fungus resistant plants would therefore provide sustainability of production of these crops to meet the demand. In addition, fungus resistant varieties require less or no fungicides and have fewer mycotoxin related problems. A way to increase antifungal resistance levels in plants is to express pathogenesis-related proteins, which plays important roles in the plant defence system. Polygalacturonase inhibitory proteins (*PGIPs*) have been shown to play such a role in plant during fungal attack (Powell *et al.*, 2000).

6.1 Regeneration of lentil

The grain legumes, in general have been considered as recalcitrant with responses to various *in vitro* techniques (Bajaj and Gossal 1981; Mroginski and Kartha 1984). In spite of this fact, several attempts have been made in regenerating plantlets from legumes including pea, chickpea, common bean, cowpea, soybean, mungbean, and peanut (Schroeder *et al.*, 1993; Jayanand *et al.*, 2002; Aragão *et al.*, 2002; Kartha *et al.*, 1981; Ikea *et al.*, 2003; Hinchee *et al.*, 1988; Jaiwal *et al.*, 2001; Wang *et al.*, 1998). Although many legumes have been regenerated using tissue culture techniques, very few efficient regeneration protocols are presently available to use them in transformation experiments.

For *in vitro* regeneration, explants were collected from aseptically germinated seeds. To get such germination, cotton or filter paper soaked with water and agar solidified medium with 2% sucrose were used (with or without MS salts). Germination on sterile distilled water soaked cotton or filter paper were observed to be faster than that on agar solidified media. Khanam *et al.* (1995) also found similar result.

Several attempts have been made in the past towards the development of a suitable protocol for *in vitro* regeneration of lentil cultivars growing in Bangladesh (Khanam *et al.,* 1995), but very limited success has been achieved. In the present investigation, a dozen various explants namely shoot-tip, epicotyl, hypocotyl, leaf, cotyledon, cotyledonary node, slited cotyledonary node, embryo, decapitated embryo, immature embryo, embryo with single cotyledon disc and LS of embryo decapitated at shoot end with single cotyledon disc were used for direct plant regeneration from them. Cotyledonary nodes from mature seeds have been most responsive for the induction of multiple shoot organogenesis in soybean, pea, pigeon pea, chickpea and *Vigna* (Cheng *et al.,* 1980; Kaneda *et al.,* 1997; Jackson and Hobbs 1990; De Kathen and Jacobsen 1990; Jayanand *et al.,* 2002; Murthy *et al.*, 1996; Subhadra *et al.,* 1998; Saini *et al.,* 2003). Among the tissues used for regeneration studies of lentil, cotyledonary explants appear to be best responding in terms of genotype independence, time duration and frequency (Warkentin and McHughen, 1993). It was also reported potential for lentil regeneration (Öktem *et al.*, 1999; Mahmoudian *et al.,* 2002). But cotyledonary node explants were found not so efficient for genetic transformation while embryo and modified embryo explants of different legumes were attracting more attention, although multiple shoot regeneration frequency is much higher in cotyledonary node explants. Use of embryo and modified embryo has been reported for pea (Schroeder *et al.*, 1993), Chickpea (Tewari-Singh *et al.,* 2004; Jayanand *et al.,* 2003). Halbach *et al.* (1998) used lentil embryo slices with decapitated root ends and also half embryos with or without cotyledon and found the latter one responding better for regeneration. Our finding was

also more or less similar to the findings of the above workers as cotyledonary node, embryo with single cotyledon disc and longitudinally sliced embryo decapitated at shoot apics with single cotyledon disc were regenerating successfully but differed in shoot number. Moreover, explants used have certain advantages as we aimed to create a direct wound surface to assist infection by *Agrobacterium*, decapitation of the shoot tip to exclude already differentiated tissue of the axillary bud. The regeneration site was thus completely exposed and easily accessible to the *Agrobacterium*. A near similar method was claimed in case of *Medicago* by Trieu and Harrison (1996) using splitting embryonic axis attached to single cotyledon. Shoot tip or cotyledonary explants contains meristemetic tissues which may be less amenable for transformation (Iglesias *et al.*, 1994; Potrykus, 1990). The decapitated CE explant used in our investigation had such tissues but the wound area was larger to make it practical for transformation.

No remarkable variation was observed among the four different varieties of lentil in case of multiple shoot regeneration except BM4 being slightly more responsive than the other three varieties, hence it was selected for the next three phases of our present investigation. Using different Bangladeshi lentil varieties Khanam (1994) also found similar response in multiple shoot regeneration. Sarker *et al.*, (2003) also found the BM2 and BM4 were responding better while they tried different lentil varieties for transformation.

A number of plant growth regulators were tested namely BAP, Kn, NAA, TDZ, IAA, GA3 for multiple shoot regeneration from the explants mentioned above. These hormones were used either alone or in combination with other hormones and also in different concentrations.

In the present study direct shoot regeneration attempts demonstrated that low concentration of BAP (2.22 - 4.44µM/l) in MS medium was most effective in regenerating multiple shoots from cotyledonary node and decapitated embryo. They were found to form healthy shoots with well developed leaves. The shoot formation was synchronized and the growth and the development of such shoots were better than those developed in other hormonal supplements. Other higher combinations of BAP also produced well developed shoots but the shoots produced in lower concentration were better in shape. This finding was similar to that finding of Khanam *et al.* (1995) who obtained responses towards multiple shoot regeneration from lentil cotyledonary node explant with 1 - 5µM/l BAP in MS medium. Higher frequency of shoot regeneration with BAP was reported by Polanco *et al.* (1988), a concentration of 10µM was mentioned as optimal multiple shoot former. The effectiveness of BA or BAP on shoot induction in lentil tissue culture has also been well documented by Saxena and King, 1987; Warkentin and McHughen 1993, Halbach *et al.* 1998, Ahmed *et al.*, 1997; Gulati and Jaiwal, 1990. Report was also been from Gulati *et al.* (2002) that 8.88 µM BA supplemented MS media was best for lentil cotyledonary node explants. In *Vigna radiata* the same concentration was also forming *in vitro* shoots from

cotyledon and hypocotyls explants (Amutha *et al.*, 2003). On the other side modified *Vigna mungo* cotyledonary node was found forming shoots on 0.5 -10µM BA containing MS with B5 vitamin media (Saini *et al.,* 2003).

TDZ (Thidiazuron), which is regarded as a 'miracle' growth regulator in plant regeneration systems, was also tried to see its effect on lentil *in vitro* regeneration. Thidiazuron is among the most active cytokinin –like substances and it induces greater *in vitro* shoot proliferation than many other cytokinins (Khawar *et al.*, 2003). Our experiments showed the number of regenerated shoots per explant increased significantly with application of TDZ compared to that of BAP. Numerous miniature shoots were regenerated from CN and DE explants in lower concentrations (0.45 - 0.908 - µM/l) TDZ. Clumps of green compact embryonic structures were induced with increased concentration of TDZ demonstrating inefficiency of the agent in shoot regeneration at higher concentrations. Halbach (1998) had similar results to ours as they used 0.23µM/l TDZ on embryo derived explants of lentil. Malik and Saxena (1992) also found similar responses with chickpea and so were Lacroix *et al.* (2003) in case of *Vigna subterranea* (Bambara groundnut). TDZ at 10 µM in lentil regeneration was reported favourable (Hassan, 2001), but TDZ at concentration of 5 µM was used in the first two to three weeks to induce normal shoot regeneration from pea (Hassan, 2006). Murthy *et al.* (1998) applied TDZ to induce a diverse array of cultural responses ranging from induction of callus to formation of somatic embryos. A mimicking character of TDZ was revealed during studies on growth and culture of explants as it was acting like both cytokinin and auxin (Murthy *et al.*, 1998; Saxena *et al.*, 1992). A number of physiological and biochemical events in cells are likely to be influenced by TDZ, since several authors reported that higher TDZ concentrations (20 µM) result in stunted shoots and consequently slow development, elongation and failure in root production (Fratini and Ruiz, 2002; Lu, 1993; Malik and Saxena, 1992). More or less similar data were reported by Khanam (1994) and Ye *et al.* (2002) that higher concentrations of TDZ inhibit shoot regeneration and produces light green to whitish shoots. The present study also confirmed the same effects of TDZ for lentil.

Combining low concentration of TDZ with BAP also induced embroid like clumps but in this case there were sudden 1 – 2 long shoots from CN and DE explants. A comparatively recent study on pea regeneration through cyclic organogenic system (Tzitzikas *et al.*, 2004) from nodal tissue demonstrates use of a number of plant growth regulators in 5 different steps till whole plant recovery. There, higher TDZ (4.99 – 9.98µM/l) or BAP (2.44- 4.88µM/l) used initially for callus like bud formation and in the next step media contained 2.27 -39.92µM/l TDZ and 1.1 – 19.52µM/l BAP for multiplication. For regenerations of shoot from these bud containing tissues B5 medium was supplemented with GA3 (2.89µM/l), NAA (5.37µM/l), and BAP (4.44µM/l).

Though they have reported a successful method but altogether it was evident from the report that it was very time consuming.

Khanam *et al.* (1995) reported that hormonal combinations and concentration (for example BAP, Kn, NAA) induced shoots but in these cases most of the shoots were not uniform in growth and development and low in number per explant. Using NAA alone did not show significant responses towards shoot regeneration in our study but it was able to form shoots when combined with BAP at low concentrations (0.54 – 2.69µM). Increase in NAA concentration was affecting the explants by forming callus like structures at the cut bases of the explants rather than forming shoots. Polanco *et al.* (1988) reported the formation of multiple shoots in MS medium with 8.88µM/l BAP and 1.07µM/l NAA in lentil. So were reporting Amutha *et al.* (2003) in case of cotyledon derived callus from *Vigna* with 1.07µM/l NAA, 8.88µM/l BA and 10% coconut water (CW). Moreover, use of BA (0.5µg ml^{-1}) and NAA (0.05µg ml^{-1}) was also mentioned to be efficient with additional $(NH_4)_2SO_4$ in B5 medium for hypocotyls explants of *Lotus japonicus* (Dasharath *et al.*, 2001).

In the present investigation combination of NAA with BAP and Kinetin was able to form shoots from different explants, in particular from cotyledonary nodes followed by decapitated embryos. Best response was with MS medium containing 2.22µM/l BAP, 2.32 µM/l Kn and 1.07 µM/l NAA. Once again it was realised that low concentrations of the hormones used were functioning better in case of *in vitro* shoot formation. Slightly contrary to this finding, Khanam (1994) achieved best response in multiple shoot regeneration on MS medium containing the same hormonal combination and concentration but supplemented with 100 mg/l CH (casein hydrolysate). As this is a rather undefined mixture, we avoided CH.

When BAP and Kn were combined together to see their effects on multiple shoot regeneration, it was found that lower concentrations of both hormones were functioning better on the previously mentioned explants. 2.22µM/l BAP and 2.32µM/l Kn were effective on CN, DE, CE explants which were considered potential explants for genetic transformation in the preliminary stage. The shoots regenerated were dwarfed though they were much healthier and stout in nature than the shoots achieved with other hormonal combinations. A near similar report on *Cajanas* (Dayal *et al.,* 2003) said 5µM/l BA and 5µM/l Kn supplemented MS media were efficient in multiple shoot regeneration from *in vitro* raised adventitious shoots. An earlier report on lentil by Williams and McHughen (1986) differed with our results as they used 46.5µM/l Kn and 0.29 or 2.89µM/l GA3 for shoots from calli derived from shoot meristem or epicotyl.

As GA3 has the characteristics of inducing cell elongation, it was added to our BAP, Kn media in combination to improve elongation of the induced *in vitro* shoots as longer shoots are one of the

major factors for *in vitro* rooting or micro-grafting. Ahmed *et al.* (1996) stresses on the use of GA3 in combination for optimal shoot growth. Comparatively longer shoots (2 - 3 cm) were obtained in our study when 0.29µM/l was used in combination with BAP and Kn. Vitrification was observed with increase of the hormone (>5.71µM/l). Using MS basal medium supplemented with GA3 (1.44µM/l) or NAA (0.27µM/l) Ye *et al.* (2002) resulted in the elongation of *in vitro* induced shoots of lentil on media containing BA or TDZ. In chickpea, use of 2µM GA3 in the shoot induction media was mentioned by Jayanand *et al.* (2003). In the same year, Amutha *et al.* also used 1.73µM/l GA3 in *Vigna radiata* and found maximum elongation of shoot. This was the similar observation by Prem Anand *et al.* (2001) for cowpea (*Vigna unguiculata*). GA3 was found efficient at a concentration of 0.58µM/l for pigeon pea *in vitro* shoot elongation (Dayal *et al.*, 2003). Except one exceptional report was from Polowick *et al.* (2004), where they were using 4.4µM/l BAP in B5 vitamin rich MS medium for elongation of embryo derived chickpea shoots. Otherwise other workers were using GA3 for shoot elongation and their findings match with our results.

In the present study, the number of shoots was maximum 6-8 per explant (CN, DE). To increase the shoot number, Tyrosine was added to our BAP, Kn, GA3 media. Since legumes are protein rich and Tyrosine is an amino acid it was thought that it may help in increasing the shoot number by promoting the shoot bud formation on the explants. Sarker and Biswas, (2002) reported on wheat embryonic calli from immature embryo explants which were producing multiple shoots on 2.22µM BAP, 2.32µM Kn and 220µM/l tyrosine containing medium. 10-12 *in vitro* shoots from lentil explants (especially CN, DE) were formed when 30.25µM of Tyrosine was used in combination; unexpectedly, the shoots were thinner and weaker but longer in comparison to the other earlier hormonal combination so far used. The observation from Sarker *et al.* (2003, 200) in lentil agrees completely with our finding.

The above observation depicts that combinations of BAP, Kn and GA3 with or without Tyrosine was efficient for multiple shoot regeneration from lentil cotyledonary node and decapitated embryo explants.

Pulses have long been considered to be recalcitrant to cell and tissue culture, with lentils among the most difficult legumes from which to generate whole plants due to problems of root induction (Fratini and Ruiz, 2002).

Attempts were taken for development of roots in the *in vitro* regenerated lentil shoots following a number of methods. Available reports indicate that root induction was achieved from media containing NAA or IAA (Polanco *et al.,* 1988) and roots were also induced in hormone free, half strength B5 medium (Warkentin and McHughen, 1993). Malik and Saxena (1992) also had results in pea using 2.5µM/l NAA but there were no mentioning about lentil or chickpea, which

were also part of that work. *V. faba in vitro* raised shoots were forming roots when cultured on RM1 (2ppm/l IBA and 1ppm/l Kn) 1st then on RM2 (2ppm/l NAA and 0.1ppm/l Kn) (Busse-Eisenreich and Kunze, 1989). 98µM/l IBA was used by Pickardt *et al. (*1995) for inducing root in *Vicia*. Ahmed *et al.* (1997) were successful in rooting with 5.37µM/l NAA in calli derived shoots of lentil which was derived from a different explant than in our rooting study. High concentration IBA (4900µM/l) pulse treatment was the only successful way among the other rooting tests carried out with IBA, NAA or IAA by Khanam *et al.* (1995).

Following the above mentioned reports, several media combinations containing various concentrations of IBA, NAA and IAA were tried to induce roots at the base of the regenerated shoots, but the media compositions used by the above workers did not showed any significant response in the present investigation. With NAA (8.06µM) or IAA (114.2µM), only succulent roots were produced. However, root induction was possible only when a high concentration (122.5µM/l) IBA was used in the media or an extremely high concentration (980µM/l) shock was given to the base of the excised regenerated shoot. The roots produced were found to be non-functional. Moreover, the shoots had to be maintained on MS medium supplemented with IBA (49µM/l). These findings regarding root formation from *in vitro* grown plants are similar to that reported by Khanam *et al.* (1994).

When in our study filter paper bridge on IBA supplemented liquid MS was used instead of agar solidified media, no significant change due to stress introduction was observed. Only one root or sometimes a bunch of secondary roots were observed to be induced from the shock induced shoots but it was very rare incident in our rooting investigation.

In a recent publication of Tzitzikas *et al.* (2004), who had also been using IAA (2.85µM) or IBA (2.46µM) or NAA (2.69µM) for rooting in pea with responses of 9%, 50% and 24% agrees with our observation. On the contrary, Khawar *et al.* (2003) reported rooting (25%) in lentil with 1.1µM/l IBA which was a comparatively lower concentration than other reports but their paper gave a sense of dissatisfaction which was followed into micro-grafting as improvement of the rooting method. A year later in a similar report was from Tewari-Singh *et al.* on chickpea rooting was obtained with 4.9µM/l IBA in 1.5% sucrose supplemented media. The same IBA concentration was reported on *V. radiata* by Amutha *et al.* (2003). Contrasting with their earlier finding, Fratini and Ruiz reported in 2003 that inverted orientation of explants on IAA (5µM/l) and Kn(1µM/l) containing media yields rooting higher than 95% in lentil. Ye *et al.* (2002) found NAA was efficient over IBA for rooting in lentil as they were successful to get 75% rooting with 8.06µM/l NAA in MS medium.

One interesting observation in our study was that the shoots were healthier and elongating well on the IBA media and *in vitro* flowering and normal seed set were there. Gulati and Jaiwal (1994) had observed more or less similar responses in mungbean.

Polanco *et al.* (1988) and Khawar *et al.* (2003) strongly comment that rooting of regenerated shoots is a major limiting factor in regenerating lentil; the views from workers on legumes suggest grafting is one of the best solutions to overcome the rooting difficulty in legumes. Gulati *et al.* (2001, 2002) found 83-96% success with micro-grafted shoots of lentil. Like Khawar *et al.* (2003) as mentioned in earlier for lentil, Tewari-Singh *et al.* (2004) also adopted grafting (72%) in case of chickpea for improvement of the regeneration protocol. So was done by Krishnamurthy *et al.* in 2000. Grafting was also reported in *Vicia* (Pickardt *et al.,* 1995), *Phaseolous* (Dillen *et al.,* 1997) *Pisum* (Böhmer *et al.,* 1995, Hassan, 2006).

Since the rooting procedure carried out using different plant hormones capable of inducing roots on *in vitro* regenerated shoots were observed to come up with only 45% success, micro-grafting was adopted in the present investigation. 58% successful grafted plants were obtained. Grafting in lentil was found to be tiresome and very difficult as the *in vitro* raised lentil shoots were thin and fragile to handle, moreover, the process was very time consuming.

But our investigation found a better solution to overcome the rooting problem as we modified our explant i.e. LS of embryo with decapitated shoot apex with a single cotyledon disc. This particular explant was found to root normally without any difficulty on MS medium devoid of any growth regulator as it had its radical portion uncut, hence the tissue culture part was also waived. This explant was also found to have potential for our next phase *Agrobacterium* – mediated genetic transformation as it had greater cut surface for the *Agrobacterium* infection. One explant was able to raise one plant only that may be the only drawback for such explant.

6.2 Genetic transformation of lentil

The second phase of the present investigation deals with the genetic transformation experiments for lentil varieties grown in Bangladesh. Comparing the different approaches, *Agrobacterium* mediated transformation has been considered as most common and successful one used in various crop plants including important grain legumes like pea, soybean etc. (Schroeder *et al.,* 1993; de Kathen and Jacobsen, 1990; Hinchee *et al.,* 1988). Protoplasts can also be used for species which cannot be transformed with *Agrobacterium* but clones, which are defined as single event transformants takes long time to become regenerated to plants. Particle bombardment is efficient method which could be targeted to any plant tissue, but it has the

unpredictability of gene integration and high risks for gene rearrangement and silencing. The method of choice therefore, is to use the natural system of *Agrobacterium*-mediated transformation since many legumes are susceptible to *Agrobacterium* infection. But it should be mentioned that till now the transformation efficiency in legumes are very low 0.03 – 5.1% (Yan *et al.*, 2000, Fontana *et al.*, 1993, Senthil *et al.*, 2004).

As a preliminary investigation, the efficiency of particular explant tissues towards transformation was investigated. The transformation ability of the explants was monitored through GUS (β- glucuronidase) histochemical assay. Various explants including cotyledonary node, decapitated embryo, and embryo with single cotyledonary disc were found to express the GUS gene following histochemical assay. ~49 transiently GUS positives from 50 CE explants, mean transformation efficiency 49.5 was obtained from four repeats. These explants showed variable nature of GUS expression. In some cases, lentil explants had much greater areas with GUS expression while in other cases only a small portion of the wounded cells were competent for transformation. Similar results regarding the expression of the GUS gene in lentil tissue have been reported earlier by Warkentin and McHughen (1992).

Agrobacterium –mediated transformation is believed to be influenced by several factors (Warkentin and McHughen, 1992; Mansur *et al.,* 1993). The efficiency of transformation and transgenic plant production depends on the establishment of a suitable protocol for inoculation, duration of co-cultivation, explant type etc. During the present investigation, keeping the OD of the bacterial suspension within the range of 0.8 – 1.2 at 600 nm, some influencing factor have been investigated, for example the incubation time during inoculation step and length of co-cultivation period. Incubation time over 45 min and not exceeding 90 min was observed to give a higher transient expression in the explants. Warkentin and McHughen (1992) were inoculating lentil epicotyl explants only for 10-15 min and they obtained some GUS positives but the paper lacks any transformation frequency information. In the same paper they also mentioned about longer co-culture period is capable of enhancing the GUS infected area. Though the inoculation time differs, their observation agrees with our results. Moreover, our GUS histochemical assays clearly revealed that the explants like cotyledonary node, decapitated embryo, embryo with single cotyledonary disc are capable at expressing the GUS gene of variable levels.

Virulence of the bacterial strain used is also a major factor in lentil as it was confirmed with four different *Agrobacterium* strains: C58, Ach5, GV3111 and A281 were tested on lentil shoot apices (Warkentin and McHughen, 1991). They have also tried strain GV2260 (p35sGUSINT) in 1992 for transformation and so have Mahmoudian *et al.* (2002). Halbach *et al.* (1998) and Hassan (2001) used EHA 101 for lentil transformation; LBA4404 was used efficiently as it was reported

by Sarker *et al.* (2003). Both groups worked with decapitated embryo explants. While working with different strains for pea transformation, Nadolska-Orczyk and Orczyk (2000) also found transformation frequencies with EHA-105 was higher than C58C1 and LBA4404. De Kathen and Jacobsen (1990) also observed similar reaction while co-cultivating epicotyl and nodal explants of pea with wild-type *Agrobacterium* strains C58C1, A281 and 8683 harbouring binary vectors GV 2260 (p35S GUS INT) and GV 3850 HPT carrying either a neomycin- or *hygromycin phosphotransferase*-gene as selectable markers. They could recover ~ 5 % of plantlets showing GUS and *NPT*II. They found that transformation frequency was influenced by explant source, *Agrobacterium* strain, genotype and duration of co-cultivation. A slightly contradictory report was from Maccarrone *et al.* (1995) for lentil where they said that the GUS gene was transiently expressed in the recipient lentil root cells in maximum after 24 hours of incubation and then decreased. We have used LBA4404 (for GUS) and EHA-105 (for *Ri-pgip*) in our study but we actually have not analyzed the transformation frequency differences between the strains for transformation of DE, CE or CN explants but in gross observation, transformation frequencies with LBA4404 were higher than with EHA-105 (transient expression only).

Regeneration of mature plants with identical phenotype and genotype is a pre-requisite for any successful transformation. Adventitious regeneration can be obtained either by somatic embryogenesis or by shoot organogenesis, and both types of regeneration can be obtained either direct or indirect via a callus phase. The direct regeneration from pre existing meristems is preferred for genetic modification in lentil. Callus based regeneration systems have the disadvantage that they have a much higher chance of yielding plants with somaclonal variations than direct regeneration. In the present study, direct shoot organogenesis was used from mature embryos after inoculation with *Agrobacterium tumefaciens* harbouring the binary vectors pSCP1 and pGIIRH0035s, containing a raspberry PGIP gene with the selectable marker gene *bar* and without *bar* gene in the latter one.

6.3 Transformation efficiency

The mean transformation efficiency of T_0 was 29.06 with a standard error of 6.02, for the transformation sets with pSCP1. Otherwise the numbers varied between 8.49 – 44.82 % while on the other side our SMF transformation with PGIIRH0035s-*Ri-pgip* showed 20.37 – 57.77% transformation rates. The result is very high compared to the results of other authors, even when one takes into account that different *Agrobacterium* strains were used and different explants and selection procedures applied. When using strain EHA105, the transformation efficiency was 3.1% in chickpea (Polowick *et al.*, 2004), 0.9 % in pea (Hassan, 2006), 10% in

Vigna mungo (Karthikeyan *et al.*, 1996) but 50% in *Vigna radiata* (Jaiwal *et al.*, 2001). Using EHA 101, the efficiency reported was 0.03% in groundnut (Cheng *et al.*, 1997). On the other hand, when strain AGL1 was used, the transformation efficiency in pea was 0.8-3.4 % (Grant *et al.*, 1995) and 1.5-2.5 % (Schroeder *et al.*, 1993). When LBA4404 was used, the efficiency was 4% in chickpea (Fontana *et al.*, 1993), 1 % in pea (Nadolska-Orczyk and Orczyk, 2000), 62% in pigeon pea (Geetha *et al.*, 1999). For lentil, recently 95% expression was reported by Mahmoudian *et al.* (2002) by using GV2260, the same strain was used by Warkentin and McHughen (1992), but transformation efficiency was not clear in their paper. Hassan (2001) had a transformation efficiency of 95.7 % and 49.9% from modified embryo explant using EHA 101 and LBA4404 respectively. His findings were in agreement with our study as in our study extra wounding treatment appeared to enhance the intensity of transformation as evidenced by GUS activity at the regeneration site. Wounding of plant material before co-cultivation possibly stimulated the production of phenolic compounds (Stachel *et al.*, 1985) and may have enhanced plant cell competence for transformation or more strongly induced the *vir*-Region or more *Agrobacteria* (Binns and Thomashow, 1988) and may have also increased transformance frequency (Bidney *et al.,* 1992).

6.4 Time to get first transgenics

We have developed a rapid transformation and regeneration system for lentil, which is significantly faster than previous methods as most of these transformation methods were coupled with regeneration via somatic embryogenesis or organogenesis through tissue culture of lentil. In our study using the modified embryo explant, it was possible to get the first transgenic plant without selection in about 2.5 months while using selection the time period increased about 2 folds. Trieu and Harrison (1996) also claimed to obtain transgenic *Medicago* plants within 2.5 months by using more or less similar explant (modified embryo explant). They also experienced that by including multiple shoot regeneration steps almost doubles the period. Polowick *et al.* (2004) found 1.3% transgenic plants with a mean tissue culture phase from co-cultivation to transfer in soil about 160 days while with the addition of shoot elongation phases, the frequency of transgenic plant recovery increased but so was the duration (217 days). The method we have used in our investigation is laborious in the initial preparation of the explants, however, the use of mature seeds is advantageous, as it does not require continuous supply of developing material. Figure 43 is showing the fast method to obtain whole lentil plantlet after transformation in short time that was used in the present study.

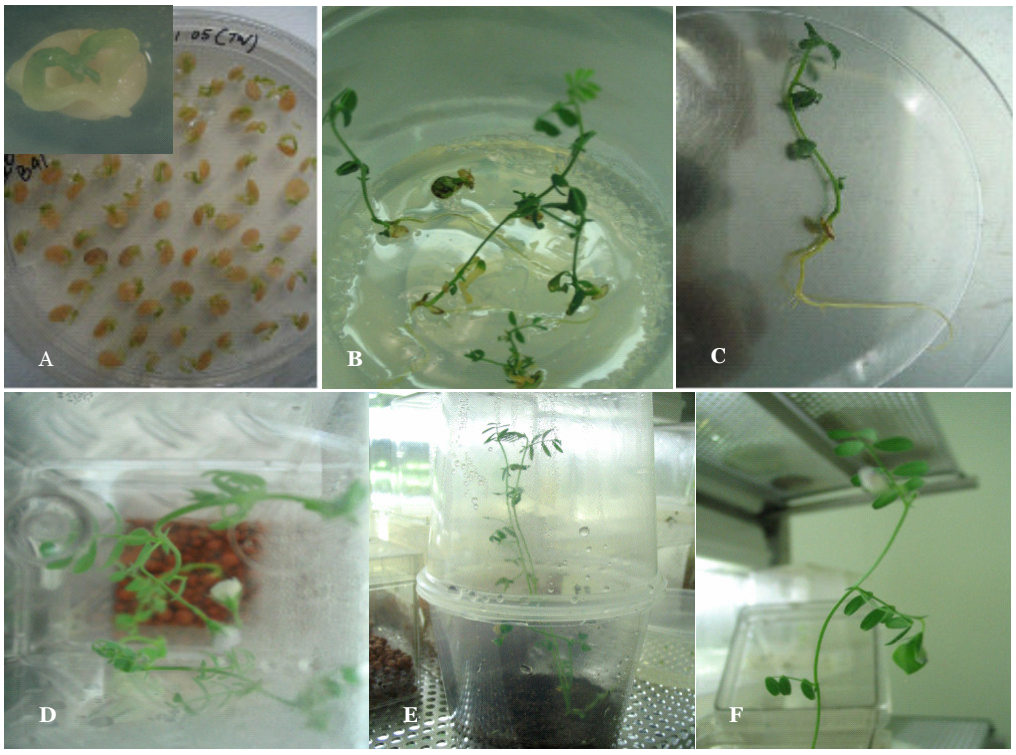


Fig. 43: The method for transformation with modified embryo explant used in the present study (A) co-culture after transformation (B) subculture on 2.5 mg/l PPT (C) plantlet from selection/free medium (D) transplantation on Seramis or (E) transplantaion on garden substrate (F) flowering and T_1 seed setting on the T_0 plants.

6.5 Selectable Marker

Selectable markers play an important role on the transformation efficiency. There are two kinds of markers used, either antibiotic or herbicide resistance. It is noticeable that the apparently higher transformation efficiencies were achieved when antibiotics were used. As it can be found in several reports kanamycin is so far the most commonly used antibiotic in lentil transformation. 10mg/l was used by Warkentin and McHughen (1992), 100mg/l (Mahmoudian *et al.*, 2002), 50mg/l Hassan (2001). In our present study, PPT (Phosphinothricin) was used for selection in transformation using the pSCP1 construct (sets with selection). Phosphinothricin is much more stringent in decreasing the rate of escapes and chimeras in comparison to kanamycin. For lentil a PPT concentration of ~2 mg/l was used by Hassan (2001) and Halbach *et al.* (1998). Also different transformation frequencies were reported using PPT and kanamycin with same plant, example in pea transformation 8.2% and 3.6 % respectively (Nadolska-Orczyk and Orczyk, 2000). This tells clearly that the transformation efficiency was higher for

kanamycin, but this result may be due to the fact that the regenerants on kanamycin were chimeric or escapes (untransformed). Also there is a report that kanamycin concentrations of more than 10mg/l caused complete root inhibition in non transformed *Vigna mungo* plants and at concentrations higher than 75 mg/l, shoot induction was inhibited completely (Saini *et al.,* 2003). This is a concern for selection and regeneration of explants after transformation. There is also a possibility of getting no transformants using kanamycin or hygromycin like antibiotics, additionally infertility may occur in the transformants as side effect of the used antibiotics (Puonti-Kaerlas *et al.*, 1992). Though we used phosphinothricin for selection, still we have observed death of flowers and either infertility of the produced seed or immature seed formation.

6.6 Marker free transformation

The issue of horizontal gene transfer (HGT) between closely, distantly or even unrelated organisms is one of the most intensively studied fields in the bio-sciences since 1940. As evidenced from the evolution studies through comparison of nucleotide sequences, it was seen that in rare case genes had been laterally transmitted amongst organisms of different domains like bacteria, archaea and eukarya (Dröge *et al.*, 1998).

Transgenic plants with herbicide resistance genes may be used as dual strategy as selective marker *in vitro* and as weed control in the field. But concerns are regarding the spread of antibiotic resistance (transformed gut bacteria) or herbicide resistance (super weed) in nature. It, therefore, is necessary, to give a second thought about the use of such selectable genes. Studies by different organizational group have not come up with any such transfer or allergenecity in GMO crops. Despite the technical evidence indicating safety, there are still political and social objections to use antibiotic resistance genes in food from raw or processed plants (Huppatz, 2000). The use of herbicide resistance as a selectable maker raises similar objections from some community groups though the primary purpose is not to exploit the herbicide resistant phenotype commercially. The presence of a herbicide resistance gene will raise the suspicion, whether justified or not, that this trait will be exploited and thereby lead to an increase in herbicide use. For bio-safety aspects, the GMOs might be more accepted by the public and easier to commercialize, especially in Europe.
Together with the above mentioned concern, a second concern of scientists related to the selectable marker is that there is frequent need to add two or more transgenes in the same plant

line in the serial transformation where it also becomes necessary to add more than one selectable marker. The availability of such markers is very limited (Scutt *et al.*, 2002).
For this reason several systems to produce selectable marker free transgenic plants have been introduced by different groups (Gelvin, 2003, Ebinuma *et al.,* 2001, Hare and Chua, 2002 Jaiwal *et al.*, 2002, Ow, 2001, Goldsbrough, 2001, Veluthambi *et al.*, 2003) and are still being improved to minimize their problems.

The systems involve (1) Simple microbial recombinase based systems (Hare and Chua, 2002, Dale and Ow, 1991). The *cre* recombinase enzyme of bacteriophage P1 has been used to excise marker genes cloned between pairs of 34 bp directly repeated *loxP* recombination sites; although such events were reported precise and leave only one *loxP* site in place, it still is limited and cannot be use with vegetatively propagated plants. Furthermore, prolonged periods of microbial recombinases may result in unwanted changes to the genome at sites removed from transgene insertion (2) Transposable element based systems (Yoder and Goldsbrough, 1994) - An engineered Maize Ac transposable element containing the *ipt* gene conferring selection through extremely shooty phenotype was conveniently been removed by such method, such an active system believed to be unreliable, and the excision of transposon from genome can alter adjacent DNA sequence (3) Co-transformation (Komari *et al.,* 1996; De Framond *et al.*, 1986) – this happens to be the choice in recent time for segregating selectable marker genes out. Here, two distinct transgene construct present in the transformed line of *Agrobacterium* are being transferred, one with the selectable marker while the other contains the desired trait gene. Gene removal is based on the principle that a portion of transformed plants carrying the selectable marker gene will also integrate the GOI at a second unlinked insertion site. Here, the method is not only unsuitable for vegetatively propagated plants, it is also asks to screen hundreds of thousand of independent transformation events to find the rare clone with both inserts. (4) An intrachromosomal recombination (ICR) system (Zubko *et al.*, 2000) – This method of removal of marker genes is based on ICR between two directly repeated sequences flanking the marker genes to be excised. A combination of lambda attachment site *att*P and negative selection using *tms*2 and napthaleneacetamide (NAM) were used for such method, but the lengthy propagation may increase the risk of somaclonal mutations. (5) The multiautotransformation (MAT) vector system (Ebinuma *et al.*, 1997; Endo *et al.,* 2002; Koichi *et al.*, 2000) – in spite it is regarded as highly sophisticated system for marker gene removal; a chosen trait gene is placed adjacent to a multigenic element flanked by recombination sites, the MAT vector system was found to incur a risk of loosing the marker gene before selection of transformed plant tissue. (6) The CLX chemically inducible system (Zuo *et al.,* 2000) – A modified Cre-*lox* system that is chemically inducible, based on a *npt*II gene positioned adjacent to a *Cre* recombinase gene driven by the

hybrid chemically inducible O^{lexA}-46 consecutive promoter and a hybrid gene XVE, encoding binding protein for *Cre* gene transcription induction, flanked by a pair of directly repeated *lox*P sites. The *Cre* and *nptII* genes are removed as a result of *Cre* recombinase activity. (7) Homologous recombination system (Iamtham and Day, 2000) – based on the homologous mechanism of plants, applicable for higher plants, deals with removal of marker genes associated with chloroplast genome, here three marker genes shares two identical promoter sequence and three identical sequence. After going through several different recombinative events, it leads to a homoplastic, marker free state. Although it operates efficiently in plant chloroplasts, homologous recombination is much less predictable and efficient when it comes for nuclear DNA. (8) *Cre-lox* recombination based systems (Srivastava and Ow, 2004; Dale and Ow, 1990; Yuan *et al.*, 2004) - basing on *Cre-lox* system this is a method to remove marker gene from chloroplast. Here, a *Cre*-recombinase gene is expressed from plant transformation cassette integrated into nuclear genome, while an N-terminal chloroplast –directing signal sequence routes the *Cre* recombinase protein that is produced to the plastids; this one like more or less all these marker excision methods require a genetic segregation step to remove the marker or the recombinase gene, however, a few exceptions are known to skip this step for example, homologous recombination and MAT vector system, furthermore, the transformation efficiency of these methods is reported to be extremely low, where the segregation step confines their use in sexually propagated plants. Finally, despite the various options for marker removal, each method is not without its limitations.

In our investigation our approach towards producing SMF plants was by constructing a small and efficient plasmid vector harbouring the gene of desired trait. The transformation is done via normal *Agrobacterium* - mediated transformation. Compared to all those selectable marker removal processes, the adopted method in the present investigation is far too simpler and efficient. It is easier as it uses the small plasmid pGreen vector with only the gene of interest, a single step transformation system includes no complex steps, requires no crossing between the transformants afterwards, selection of the transformants is also very easy as they can be selected through PCR or RT–PCR. Moreover, as observed in the present investigation, the transformation efficiency is also relatively high. And above all, supporting the bio-safety concern it may be a competent system to be used for crop improvement and commercialization in the future.

6.7 *Ri-pgip* gene

In this study, the raspberry PGIP1 gene (*Ri-pgip),* which was cloned by Ramanathan *et al.* (1997), was used in order to increase disease resistance of plants against fungal pathogens. Raspberry PGIP was identified and isolated by Johnston *et al.* in 1993; the protein is a single polypeptide chain with *Mr* of 38-5 kDa and a p*I* residing above pH 10. The characteristic of the PGIP is similar to the PGIP from *P. vulgaris* (Cervone *et al.,* 1987). Both proteins have a similar molecular weight on SDS-PAGE and similar specificity towards fungal and bacterial PGs (Cervone *et al.,* 1987, 1989); both proteins inhibit endo-PGs from fungi and are ineffective against PGs and PLs from bacteria. PGIP from raspberry has 44% similar amino acid sequence identity with *Phaseolus vulgaris* L. The PGIP from raspberry is ionically bound to cell walls (88%). Ramanathan *et al.*, (1997) cloned two different cDNA from raspberry. The 1325 bp full length PGIP1 cDNA contained an open reading frame predicted to encode a 331 amino acid protein. Also a second PGIP cDNA (PGIP2) was cloned and sequenced from the raspberry library. Comparison of PGIP1 and PGIP2 shows 82% identity at the nucleotide sequence level and reserves the characteristics of PGIPs at the amino acid level. The raspberry PGIP contains four potential N-glycosylation sites (N-T/S), three of which show a conserved position with previously isolated PGIP genes. Two potential phosphorylation sites described for *Antirrhinum* PGIP (Steinmayr *et al.,* 1994) are absent in the predicted raspberry peptide. PGIP1 shows high leucine content (15.7%) and contains 10 loosely conserved leucine-rich repeats (P L--L--L-LSN-L-G-I) (Stotz *et al.,* 1994). Southern analysis of cDNA of raspberry by Ramanathan *et al.* (1997) suggests that raspberry PGIPs are members of a low copy number gene family. Besides these two genes (PGIP1, PGIP2), a third clone (1136 bp in length) which showed variation from PGIP1 in the 5' and 3'untranslated region only, may suggest at least three copies of PGIP in raspberry, PCR analysis between PGIP1 and PGIP2 shows that these genes are closely linked (Ramanathan *et al.,* 1997). They also reported that the raspberry PGIP gene contains an intron; an efficiently spliced 243 bp intron was identified that shows a high AT content (70% AT). Raspberry PGIP maintains all of the structural features observed in the previously cloned PGIP genes (Pear, Kiwi, Tomato, *Antirrhinum,* Bean and Soybean).

Ri-pgip gene was cloned into the pGreenII vector under control of the double 35s cauliflower mosaic virus (CaMV) RNA promoter. The 35S promoter has properties that make it useful in transgenic crop development because of constitutively rather high levels of gene expression activity in many plant cells. It is one of the best-studied elements controlling gene expression in plants. The *nos* promoter was considered to be weaker than the 35S promoter and its activity is organ-, position- and developmental-stage dependent, taking into consideration the fact that

nos promoter activity is differentially expressed in various organs, which indicates potential problems in regenerating transformants. The activity of the *nos* promoter differs between different plant species (An *et al.*, 1987 and 1988). Sanders *et al.* (1987) compared the CaMV 35S promoter and the *nos* promoter at transcriptional levels in transgenic petunia plants, where they found that 35S promoter was at least 30 times stronger than the *nos* promoter. Harpster *et al.* (1988) had found similar data. However, there are some disadvantages of using 35S promoter as it shows morphological, developmental and physiological alterations in the transgenic plants (Fladung *et al.*, 1997). Functionality of the binary vector and cloned *Ri-pgip* gene were tested and lentil was used as legume model plant.

6.8 Molecular characterization

In our present investigation the integration of the transgenes for both constructs was confirmed with PCR. For our gene of interest, the *Ri- pgip* gene, mainly two primer sets were used, with product sizes 365 bp and 750 bp. For the selectable marker gene *bar* one primer set with product size 447 bp. Most of the T_o plants were positive for GOI and *bar*. Altogether giving 29.06 ± 6.02 SE as transformation rate with the pSCP1 construct. This is a rather high transformation rate as that normally has been reported for *Agrobacterium* mediated legume transformation to be between 0.03% (ground nut, Cheng *et al.*, 1997) –4.9%(pea, de Kathen and Jacobsen, 1990). In some cases we found that although the T_o clone or the T_1 plants were positive with *Ri-pgip* gene but some of them turned out negative in the PCR analysis for the *bar* gene.

It is well known that T-DNA transfer to plant cells occurs in a defined direction, starting from the right border to the left border (Becker *et al.*, 1992; Zambyski, 1992), where the selectable genes are located to ensure selecting transformants containing complete T-DNA insertions. There have been reports (like in the present work) that PCR for the *bar* gene often runs negative. Hassan (2006) has reported a similar phenomenon when he was transforming pea. Findings by Richter (2004) are also in agreement with the results in the present study. This could be the result of incomplete T-DNA transfer as the transfer initiated from the right border got aborted before reaching the left border. As the location of the *bar* gene is near to the left border in the pSCP1 construct used, likely the *pgip* gene was transferred completely but not the *bar* gene. Since the *bar* gene is only for selection of the transformants carrying the gene of interest, the functionality assay may help to overcome such problems because a negative *bar*

PCR does not mean that the plant is not transgenic. In contrast, this may be beneficial, as our study, anyway, aims for a selectable marker free system.

To support the above statement, in the present investigation transformants with the novel pGreen vector containing the *Ri-pgip* gene only have been confirmed with PCR for GOI as it lacks any selectable marker gene. In this set of experiment the mean transformation success rate obtained was 35.57 ± 11.34 SE. Further confirmation was done through functional gene expression analysis via PGIP assays to eliminate any spurious amplification.

6.9 Backbone analysis

In the present investigation transformants obtained with the pSCP1 construct were mostly 'clean', but few of the transformants carried the backbone sequences. Out of 82 T_0 clones (EX 17, 18), 9 clones had integrated with backbone sequences of the vector. That corresponds to 10.97%. The proof of backbone sequence presence in the plant genome was determined in our study by means of PCR.

For many years it was believed that with *Agrobacterium* mediated gene transfer only the sequence (T-DNA) between the two borders is integrated into the plant genome. Detailed analysis of gene transfer in plants has however shown that the integration of vector sequences into the plant genome very frequently takes place (Ramanathan *et al.*, 1995; Van der Graaff *et al.*, 1996). The first report of such transfer was by Martineau *et al.,* (1994). Transfer of non-TDNA portion at fairly high frequencies was seen when the transfer initiates from right border but skips the left border and results in the transfer of the whole binary vector into the plant genome. T-DNA transfer occurs in low frequency from left border as well (Veluthambi *et al.*, 2003). This is a potential biohazard because the presence of uncharacterized DNA in transgenic plants is important for regulatory concerns. These events should be identified and eliminated.

Recently, it was also shown that the new vector system like pGreen/pSoup, the so called 'small vectors', also bear the risk of transferring backbone sequences in noticeable frequencies. Vain *et al.*, (2003) in their work with transgenic rice detected 45 % of the lines with multiple copy insertion carrying backbone, while only 15-20 % of the lines with single copy T-DNA integration without backbone. The overall expression did not improve with increasing the copy number of T-DNA. Vector backbone sequence, were also observed by Yin and Wang (2000) in rice 33%.

6.10 Functional assay

6.10.1 Leaf paint

Successful expression and functionality of the *bar* gene was tried to be confirmed by the leaf-painting assay. A number of concentrations of BASTA® were used in the present study to optimize the applicable concentration for lentil. There is so far no report on lentil leaf paint assay. The concentration of BASTA® used was compared with other legumes especially with pea, where usual concentrations are 3 mg ml $^{-1}$ (Bean *et al.*, 1997), 200 mg/l (Nadolska-Orczyk and Orczyk, 2000), 600 mg/l on pea (Hassan, 2006) or 400 mg/l on Faba bean (Hanafy *et al.*, 2005). Herbicide tolerance gives another advantage for the transformed plants as they can survive when the same herbicide is used to control weeds. It was found that most of the lentil clones were extremely sensitive to BASTA. Even 37.5 mg/l affected the plants which have already been shown to have integrated the *bar* gene (PCR data). By applying the leaf paint assay, the transgenic plants could be discriminated from non-transgenic plants, by exhibiting the resistance against the total herbicide BASTA® (37.5 mg/l PPT), whereas non-transgenic plants showed necrosis and the treated parts or the whole leaf turned yellow and died. Due to possible gene silencing phenomena, the herbicide sensitive plants are not necessarily non-transgenic and due to this fact the leaf paint assay only allows positive selection.

Despite the T_1 plants showing negative leaf paint result as in the case of T_0 clone 14-15 (which was positive in PCR for *bar* gene), this may be due to gene inactivation, methylation or co-suppression (D' Halluin *et al.*, 1992) or due to the physical loss of the gene due to incomplete T-DNA transfer to the plant genome, since the *bar* gene is located next to the GOI (*Ri-pgip)* gene near the left border (Hassan, 2006). This can explain negative PCR results for *bar* and positive one for *Ri-pgip* in some of our transformants (data not shown). The chimeric character of some T_0 plants could also be one of the reasons for the BASTA® sensitivity. The expression level proved to be varying between different clones from the same transformant and even between plants from one clone “inter individual differences” (Richter, 2004).

T-DNA could integrate near to far from transcriptional activating elements or enhancers, resulting in the activation or lack of activation of the transgene. It can also get integrated in transcriptionally silent regions of the plant genome. Linked and unlinked copies of introduced genes and related endogenous genes in plants can be silenced by homology-based mechanisms at the transcriptional (TGS) or post-transcriptional level (PTGS), through DNA methylation or unstable RNA after transcription (Matzke and Matzke, 1998; Veluthambi *et al.*, 2003; Gelvin, 2003). In addition, the expression level can be affected by adjacent plant DNAs or the different sequences flanking the integration sites leading to as “position effects” (Hobbs *et al.*, 1990;

Finnegan and McElory, 1994). Some authors concluded positive correlation of copy numbers with gene silencing (Klimaszewska *et al.,* 2003). Hobbs *et al.* (1990) found that two allelic copies of T-DNA resulted in doubling the expression, whereas non-allelic copies reduced the expression. Integration of T-DNA repeats especially 'head to head' inverted repeats around the T-DNA right border, often resulted in transgene silencing (Cluster *et al.*, 1996). Any one of these may be the explanation for the inefficiency of the introduced *bar* gene in the T_1 progeny in this study, because copy number of the insert was not analyzed in our study.

6.10.2 PGIP assay

The successful expression and function of the fungus resistant gene *Ri-pgip* from raspberry was studied through PGIP assays by using crude total protein from T_1 – T_2 plants of the T_0 clones from transformation with pSCP1 where the T-DNA contained both genes, the GOI, *Ri-pgip,* and the selectable marker gene *bar*. On the other side from the transformation with the marker free construct (PGII) that contains only the GOI, only the T_0 were subjected for PGIP assay. In the present study, leaf samples were used for crude protein extraction since the 35S promoter confers high levels of expression in leaves and stems of transgenic plants and lower expression in flowers and seeds (Malik *et al.*, 2002). A total protein amount of 20µg was efficiently inhibiting the introduced fungal PGs (polygalacturonases). Lower concentrations had reduced inhibition effects. The T_1 plants were found strongly inhibiting PGs from *Botrytis* while working slightly less efficiently against *Colletotrichum lupini* which was comparatively stronger fungi than *Botrytis*. The *Ri-pgip* was found completely inefficient against *Colletotrichum acutatum* PG, which was considered strongest among the PGs subjected in the investigation. The T_0 plants from our marker free transformation were efficiently inhibiting PG from *Ascochyta*. The samples subjected for PGIP assay in our study were all positive in the PCR for GOI. In some cases there were no or very negligible inhibition effect observed in extracts from plants in the PGIP assay. This could be due to expression instability as the lentil itself should be having *pgip* genes. The homology of the recombinant gene would be more crucial to the *pgip* gene from lentil, but so far no sequence homology for lentil endogenous *pgip* was reported. With the remarks of De Lorenzo *et al.* (2001), the group of *pgips* sequenced so far from legumes are clearly distinguishable from the other *pgips*, raspberry *pgip* is also among these. High homology between two genes may lead to gene silencing effect which was seen in tobacco (Kunz *et al.,* 1996).

A varied inhibition response was observed between the T_1 plants of a single T_0 clone (14-15). These particular T_1 plants were positive in their PCR analysis for the GOI and the *bar* gene and

were found negative in leaf paint assay. They were found showing varied positive inhibition expression in the PGIP assay. The variance in the expression could be due to the influence of external factors, which can not be overlooked completely. The plants, however, were cultured under the same conditions and there were no apparent differences in their development. The variable expression within population is already a topic in the population genetics (Rasmusson, 1996). Similar expression variation was observed by De Neve *et al.* (1999) while they were working with 5 different *Arabidopsis* lines and also by Richter *et al.* (2006) with peas.

In support of the successful integration of the transgene, our T_0 clones from the Marker-free transformation lot were confirmed for the GOI with PCR and PGIP. For instance clone 19-1 was confirmed positive in the PCR for *Ri-pgip* but in the PG assay it was found almost non-inhibiting with 0.48%, while PCR positive clone 19-18 was inhibiting at 80.14%. This phenomena can only be explained as differential expression of the integrated gene or silencing due to TGS or PTGS or any other reason that were discussed earlier in the case of the non-expressing *bar* gene or it could be due to the very specific PG/PGIP interaction (De Lorenzo *et al.*, 2001).

6.11 Out look

In the present study, different transgenic lentil clones could be obtained from different binary vectors. These plants expressed anti-fungal *Ri-pgip* gene, the T_2 -T_3 progeny need to be analysed.

Transcription and segregation analysis of these plants are necessary to establish a single copy line. Also it is necessary to continue multiplication of the selected clones in order to establish homozygous lines.

Since the crude extract could inhibit different fungal polygalacturonases, it will be effective to test the anti-fungal effect *in vivo* under field conditions with different fungi (pathogen challenging).

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STATEMENT

I, hereby, declare that I composed this thesis myself. I did not use other auxiliary material than indicated. Other work has been always cited.

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Rehana Hashem